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CONTENTS OF VOLUME 50

NO. 1, JANUARY, 1952

INGLE, DWIGHT J., JAMES E. NEZAMIS AND ERVING H. MORLEY
The Comparative Value of Cortisone, 17-hydroxycorticosterone and Adrenal Cortex Extract by Continuous Intravenous Injection in Sustaining the Ability of the Adrenalectomized Rat to Work 1

COHEN, HERMAN AND ROBERT W. BATES
The Demonstration of a Non-estrogenic Uterine Stimulating and Estrogen Augmenting Substance in Pregnant Mares' Urine 5

KNOBIL, ERNEST
The Relation of Some Steroid Hormones to β-Glucuronidase Activity 16

GUIDRY, MARION A., ALBERT SEGALOFF AND AARON M. ALTSCHUL
The Effect of a-Estradiol, Some of its Esters and Estrone on the Respiration of Tissue Homogenates 29

MENKES, JOHN H. AND ARPAD CSAPO
Changes in the Adenosine Triphosphate and Creatine Phosphate Content of the the Rabbit Uterus Throughout Sexual Maturation and after Ovulation 37

ROBERTS, KATHLEEN E. AND ROBERT F. PITTS
The Influence of Cortisone on Renal Function and Electrolyte Excretion in the Adrenalectomized Dog 51

SULMAN, F. G.
Intravaginal Assay of Estrogens 61

BOTKIN, A. L. AND H. JENSEN
The Effect of Epinephrine and Thyrotropin on Thyroid Function in Rats 68

SOURKES, T. L. AND P. HENEAGE
Some Studies on the Oxidative Metabolism of the Adrenal Gland 73

KITCHELL, RALPH L. AND L. J. WELLS
Reciprocal Relation Between the Hypophysis and Adrenals in Fetal Rats: Effects of Unilateral Adrenalectomy and of Implanted Cortisone, DOCA and Sex Hormones 83

GRAD, B.
A Simple Method for the Measurement of the Oxygen Consumption and Heart Rate of Rats 94

ENGEL, FRANK L., ALBERTO VIAU, WILLIAM COGGINS, AND WILLIAM S. LYNN, Jr.
Diabetogenic Effect of Growth Hormone in the Intact Force-fed Adrenocorticotrophin Treated Rat 100

GOLDBERG, R. C. AND I. L. CHAIKOFF
Myxedema in the Radiothyroidectomized Dog 115

PEREIRA, J., JR.
Transient Oxytoxic Depression of Immature Guinea Pig Fed on Grass 124

BOATMAN, JOSEPH B., CLEM RUSS, JOSEPH H. SUNDER, ANDREW KONNERTH AND CAMPBELL MOSES
I^{131} Distribution in Normal and Hemithyroidectomized Rats Treated with Desoxycorticosterone Acetate 134

NOTES AND COMMENTS
HALMI, N. S.
Two Types of Basophils in the Rat Pituitary: "Thyrotrophs" and "Gonadotrophs" VS. Beta and Delta Cells 140

NO. 2, FEBRUARY, 1952

SHEPHERD, STANLEY J., JR., MILLARD J. SMITH AND BERNARD B. LONGWELL
The Effect of Alloxan Diabetes on the Response of the Adrenal Gland to Cold Stress 143

SIMPSON, S. A. AND J. F. TAIT
A Quantitative Method for the Bioassay of the Effect of Adrenal Cortical Steroids on Mineral Metabolism 150

TEPPERMAN, JAY AND HELEN M. TEPPERMAN
Effects of Purified Gonadotrophins on the Cholesterol Content of the Testes of Immature Rats 162

KLITGAARD, H. M., H. B. DIRKS, JR., W. R. GARLICK AND S. B. BARKER
Protein-Bound Iodine in Various Tissues After Injection of Elemental Iodine 170

BLOODWORTH, J. M. B., JR.
The Acute Effect of Adrenal Cortical Extract, Adrenocorticotropic Hormone, and

Desoxycorticosterone Acetate on the Thiocyanate, Bromide, and Mannitol Spaces; Plasma Volume and Total Body Water in Normal Dogs 174
HAYASHIDA, T. AND CHOH HAO LI
Enhancement of Adrenocorticotropic Hormone Activity by Alum in Normal 21-Day Old Male Rats 187
WEISS, SAMUEL B., LORE MARX AND WALTER MARX
The Effects of Thyroid and of Choline and Inositol on Cholesterol Distribution in Rats Fed a High Cholesterol Diet 192
LEONARD, SAMUEL L.
A Glycostatic Effect of Testosterone on the Perineal Muscles of the Rat 199
HOFMANN, FREDERICK G.
Water Excretion in Hypophysectomized Rats Before and After Adrenalectomy .. 206
BEACH, ELIOT F., PHOEBE J. BRADSHAW AND N. R. BLATHERWICK
Response to Alloxan Modified by Gonadectomy 212
PETERSON, ROY R. AND WILLIAM C. YOUNG
The Problem of Placental Permeability for Thyrotropin, Propylthiouracil and Thyroxine in the Guinea Pig 218
GESCHWIND, IRVING I. AND CHOH HAO LI
Inactivation of Adrenocorticotropic Hormone in Vitro by Tissues 226
ADAMS, A. ELIZABETH AND JO ANNE M. BUSS
The Effect of a Single Injection of an Antithyroid Drug on Hyperplasia in the Thyroid of the Chick Embryo 234
JACOT, BERNARD AND HANS SELYE
On Extra-Adrenal Actions of Adrenocorticotrophic Preparations 254
HERRICK, E. H., E. RUTH MEAD, BARBARA W. EGERTON AND J. S. HUGHES
Some Effects of Cortisone on Vitamin C-Deficient Guinea Pigs 259
BACCHUS, HABEEB, MELVIN H. HEIFFER AND NORMAN ALTSZULER
Cytochemical Study of the Adrenal Cortex of the Rat with Experimental Cardiac Hypertrophy 264
NOTES AND COMMENTS
KIRGIS, HAROLD AND IRVING ROTHCHILD
The *In Vivo* Inactivation of Estradiol by the Human Liver 269
ZONDEK, BERNHARD AND MICHAEL FINKELSTEIN
Excretion of Estrogens and a Metabolite in the Urine of Female Pseudo-hermaphrodites 271
ALBERT, A. AND ELIZABETH FORD
The Effect of Iodothiouracil on the Biologic Decay of Thyroidal Radioiodine 272
FARRELL, G. L. AND S. M. MCCANN
Detectable Amounts of Adrenocorticotrophic Hormone in Blood Following Epinephrine 274

NO. 3, MARCH, 1952

MORRIS, DAVID M.
The Thyrotrophic Activity of the Pituitary of the White Leghorn Cockerel as Related to its Cytology 277
MARCUS, FRANK, LOUISE P. ROMANOFF AND GREGORY PINCUS
The Electrolyte-Excreting Activity of Adrenocortical Substances 286
MCSHAN, W. H., AND ROLAND K. MEYER
Gonadotrophic Activity of Granule Fractions Obtained from Anterior Pituitary Glands of Castrate Rats 294
MCARTHUR, JANET W.
The Identification of Pituitary Interstitial Cell Stimulating Hormone in Human Urine 304
LEATHEM, JAMES H., AND LESLIE A. STAUBER
Adrenal Lipid and Alkaline Phosphatase in Hamsters Infected with Leishmania Donavani 311
MAYER, JEAN, RUTH E. RUSSELL, MARGARET W. BATES, AND MARGARET M. DICKIE
Basal Oxygen Consumption of Hereditarily Obese and Diabetic Mice 318
ALBERT, A., ALIDA TENNEY AND ELIZABETH FORD
The Effect of Cortisone and Corticotropin on the Biologic Decay of Thyroidal Radioiodine 324
ALBERT, A., ALIDA TENNEY AND NONA LORENZ
The Effect of Hypophysectomy on the Renal Clearance of I^{131} 327
GROLLMAN, ARTHUR
The Maintenance of the Adrenalectomized Dog for Prolonged Periods Without Recourse to Hormonal Therapy 331

SELIGMAN, ARNOLD M. AND RIVKA ASHBEL
Histochemical Demonstration of Ketosteroids in Adrenal Cortical Tumors with or without an Associated Cushing's Syndrome 338
AMES, ROSE G. AND H. B. VAN DYKE
Antidiuretic Hormone in the Serum or Plasma of Rats 350
KALMAN, SUMNER M.
The Effect of Androgens on the Succinoxidase System of Rat Liver 361
SAVARD, KENNETH, WILLEM J. KOLFF AND ARTHUR C. CORCORAN
Corticosteroids of Peripheral Blood 366
NOTES AND COMMENTS
ALBERT, A., ALIDA TENNEY AND NONA LORENZ
The Absorption of Thyroxine from the Gastrointestinal Tract of the Rat .. 374

NO. 4, APRIL, 1952

WINTERS, R. W., R. B. SCHULTZ AND W. A. KREHL
The Adrenal Cortex of the Pantothenic Acid-Deficient Rat: Eosinophile and Lymphocyte Responses 377
PERRY, W. F. AND G. R. CUMMING
Adrenal Succinic Dehydrogenase Activity Determined by the Reduction of Tetrazolium Salt by Adrenal Homogenate 385
WINTERS, R. W., R. B. SCHULTZ AND W. A. KREHL
The Adrenal Cortex of the Pantothenic Acid-Deficient Rat: Carbohydrate Metabolism 388
GEMZELL, CARL A.
Decrease in the Formation of Adrenocorticotropin Following Administration of Epinephrine 399
GRAYHACK, JOHN T. AND WILLIAM WALLACE SCOTT
The Effect of General Dietary Deficiencies on the Response of the Prostate of the Albino Rat to Testosterone Propionate 406
BURSTEIN, SHLOMO
The Guinea Pig as a Laboratory Animal for Corticoid Excretion Studies: Modification of the Formaldehydogenic Method 412
ZONDEK, BERNARD AND SHLOMO BURSTEIN
The Relationship of Corticoid Excretion to Ovarian Hormones in the Guinea Pig 419
SZEGO, CLARA M.
Pituitary-Adrenal Cortical Antagonism to Estrogenic Stimulation of the Uterus of the Ovariectomized Rat: Observations on Structural Specificity of Crystalline Steroids 429
PERLMUTTER, MARTIN, SHIRLEY WEISENFELD AND MONROE MUFSON
Bioassay of Insulin in Serum Using the Rat Diaphragm 442
MARDEN, WILLIAM G. R.
The Hormone Control of Ovulation in the Calf 456
ENGEL, FRANK L.
On the Nature of the Interdependence of the Adrenal Cortex, Non-Specific Stress and Nutrition in the Regulation of Nitrogen Metabolism 462
HALL, C. E. AND O. HALL
The Effect of Cortisone on the Course of Parabiotic Hypertension in the Rat 478
ROTHCHILD, IRVING AND ALLAN C. BARNES
The Effect of Dosage, and of Estrogen, Androgen or Salicylate Administration on the Degree of Body Temperature Elevation Induced by Progesterone 485

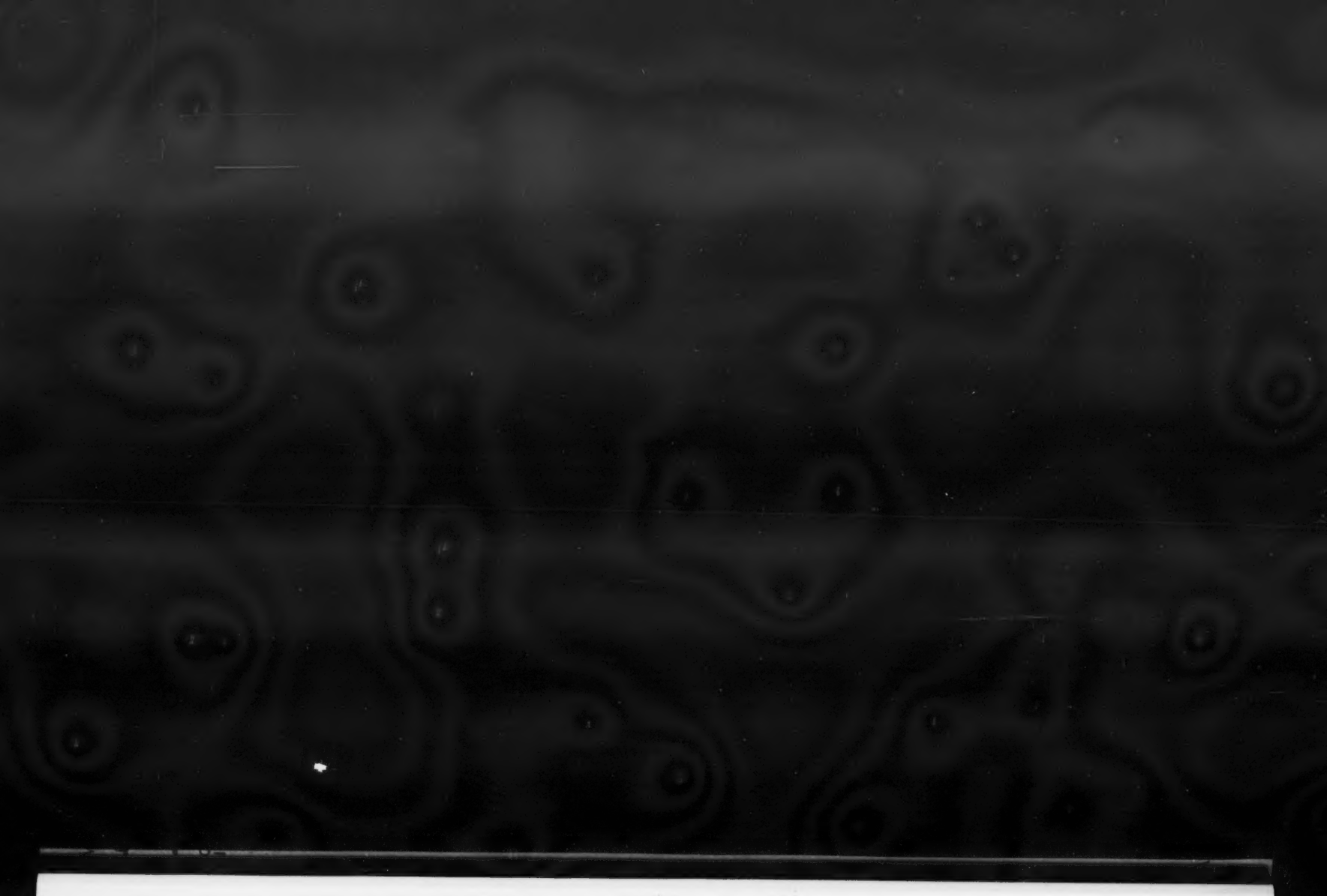
NO. 5, MAY, 1952

BEYER, ROBERT E.
The Effect of Thyroxin Upon the General Metabolism of the Intact Chick Embryo 497
SCHILLER, SARA, EARL P. BENDITT AND ALBERT DORFMAN
Effect of Testosterone and Cortisone on the Hexosamine Content and Metachromasia of Chick Combs 504
WEEKS, M. H., J. KATZ AND N. G. FARNHAM
Organic and Inorganic Iodine Levels in Serum of Sheep Receiving Daily Oral Doses of I^{131} 511
GAUNT, ROBERT, JAMES H. LEATHEM, CONSTANCE HOWELL AND NANCY ANTONCHAK
Some Biological Properties of Different Esters of Desoxycorticosterone 521
WEXLER, BERNARD C.
Adrenal Ascorbic Acid Changes in the Gonadectomized Golden Hamster Following a Single Injection of Diethylstilbestrol 531

WYNGAARDEN, JAMES B., BENJAMIN M. WRIGHT AND PETER WAYS
The Effect of Certain Anions Upon the Accumulation and Retention of Iodide by the Thyroid Gland 537
BOTKIN, A. L., E. H. PRAYTOR, M. E. AUSTING AND H. JENSEN
Thyroid Response to Total Body X-Irradiation 550
ZARROW, M. X. AND J. T. BALDINI
Failure of Adrenocorticotropin and Various Stimuli to Deplete the Ascorbic Acid Content of the Adrenal Gland of the Quail 555
RATHER, L. J.
Constrictive Renal Hypertension in Rats Having Adrenal Autotransplants with Hepatic Portal Drainage 562
ROBERTSON, THEODORE
A Sex Difference in the Tolerance of Wistar Rats for Potassium 569
TALALAY, PAUL, MARIE MOLLOMO DOBSON, CARL M. EBERSOLE AND CHARLES HUGGINS
Physiological Interaction of Cortisone Acetate and Diethylstilbestrol. Lack of Androgenic or Estrogenic Effects of Cortisone Acetate 574
NOTES AND COMMENTS
ROTHCHILD, IRVING AND RICHARD L. RAPPORT
The Thermogenic Effect of Progesterone and Its Relation to Thyroid Function 580
ROTHCHILD, IRVING
A Simple Method for the Preparation of Aqueous Solutions of Steroid Hormones for Intravenous Administration 583
SCOTT, J. KIMBALL
The Effect of Nor-Epinephrine on the Eosinophil Count in the Mouse 585

NO. 6, JUNE, 1952

BARRY, MICHAEL C., MAXWELL LEIGH EIDINOFF, KONRAD DOBRINER AND T. F. GALLAGHER
The Fate of C^{14}-Testosterone and C^{14}-Progesterone in Mice and Rats 587
BROWN, MINA MACNAIR AND WILLIAM C. YOUNG
The Effects of Propylthiouracil on the Growth of Young Male Guinea Pigs 600
SCOTT, CHARLES C., ROBERT L. KROC AND NEIL R. STASILLI
Metabolic and Toxicity Studies on Para-Hydroxypropiophenone 607
MALOOF, FARAHE, BROWN M. DOBYNS AND AUSTIN L. VICKERY
The Effects of Various Doses of Radioactive Iodine on the Function and Structure of the Thyroid of the Rat 612
SAFFRAN, MURRAY, BERNARD GRAD AND MARGARET J. BAYLISS
Production of Corticoids by Rat Adrenals *in Vitro* 639
BROWN, E. M. JR., F. C. DOHAN, L. R. FREEDMAN, P. DE MOOR AND F. D. W. LUKENS
The Effects of Prolonged Infusion of the Dog's Pancreas with Glucose 644
WHITNEY, JOHN E. AND LESLIE L. BENNETT
Inhibition of the Catabolic Effect of Adrenocorticotropic Hormone (ACTH) in Rats by a Diet High in Potassium Chloride 657
GREEP, ROY O., ERNEST KNOBIL, FREDERICK G. HOFMANN AND THOMAS L. JONES
Adrenal Cortical Insufficiency in the Rhesus Monkey 664
BRISTOL, WILLIAM R., AND VICTOR A. DRILL
Saline Hydration and Diuresis after Adrenalectomy 677
NOTES AND COMMENTS
GLAFKIDES, CONSTANTINE M., LESLIE L. BENNETT AND ROBERT GEORGE
Inhibition of the Diabetes Enhancing Effect of ACTH by a Diet High in Potassium Chloride 684
INDEX 687



Volume 50 JANUARY, 1952 Number 1

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ENDOCRINOLOGY

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INGLE, DWIGHT J., JAMES E. NEZAMIS AND ERVING H. MORLEY
The Comparative Value of Cortisone, 17-hydroxycorticosterone and Adrenal Cortex Extract by Continuous Intravenous Injection in Sustaining the Ability of the Adrenalectomized Rat to Work 1

COHEN, HERMAN AND ROBERT W. BATES
The Demonstration of a Non-estrogenic Uterine Stimulating and Estrogen Augmenting Substance in Pregnant Mares' Urine 5

KNOBIL, ERNEST
The Relation of Some Steroid Hormones to β-Glucuronidase Activity 16

GUIDRY, MARION A., ALBERT SEGALOFF AND AARON M. ALTSCHUL
The Effect of a-Estradiol, Some of its Esters and Estrone on the Respiration of Tissue Homogenates 29

MENKES, JOHN H. AND ARPAD CSAPO
Changes in the Adenosine Triphosphate and Creatine Phosphate Content of the Rabbit Uterus Throughout Sexual Maturation and after Ovulation 37

ROBERTS, KATHLEEN E. AND ROBERT F. PITTS
The Influence of Cortisone on Renal Function and Electrolyte Excretion in the Adrenalectomized Dog 51

SULMAN, F. G.
Intravaginal Assay of Estrogens 61

BOTKIN, A. L. AND H. JENSEN
The Effect of Epinephrine and Thyrotropin on Thyroid Function in Rats 68

SOURKES, T. L. AND P. HENEAGE
Some Studies on the Oxidative Metabolism of the Adrenal Gland 73

KITCHELL, RALPH L. AND L. J. WELLS
Reciprocal Relation Between the Hypophysis and Adrenals in Fetal Rats: Effects of Unilateral Adrenalectomy and of Implanted Cortisone, DOCA and Sex Hormones 83

GRAD, B.
A Simple Method for the Measurement of the Oxygen Consumption and Heart Rate of Rats 94

ENGEL, FRANK L., ALBERTO VIAU, WILLIAM COGGINS, AND WILLIAM S. LYNN, JR.
Diabetogenic Effect of Growth Hormone in the Intact Force-fed Adrenocorticotrophin Treated Rat 100

GOLDBERG, R. C. AND I. L. CHAIKOFF
Myxedema in the Radiothyroidectomized Dog 115

PEREIRA, J., JR.
Transient Oxytoxic Depression of Immature Guinea Pig Fed on Grass 124

BOATMAN, JOSEPH B., CLEM RUSS, JOSEPH H. SUNDER, ANDREW KONNERTH AND CAMPBELL MOSES
I^{131} Distribution in Normal and Hemithyroidectomized Rats Treated with Desoxycorticosterone Acetate 134

NOTES AND COMMENTS

HALMI, N. S.
Two Types of Basophils in the Rat Pituitary: "Thyrotrophs" and "Gonadotrophs" VS. Beta and Delta Cells 140

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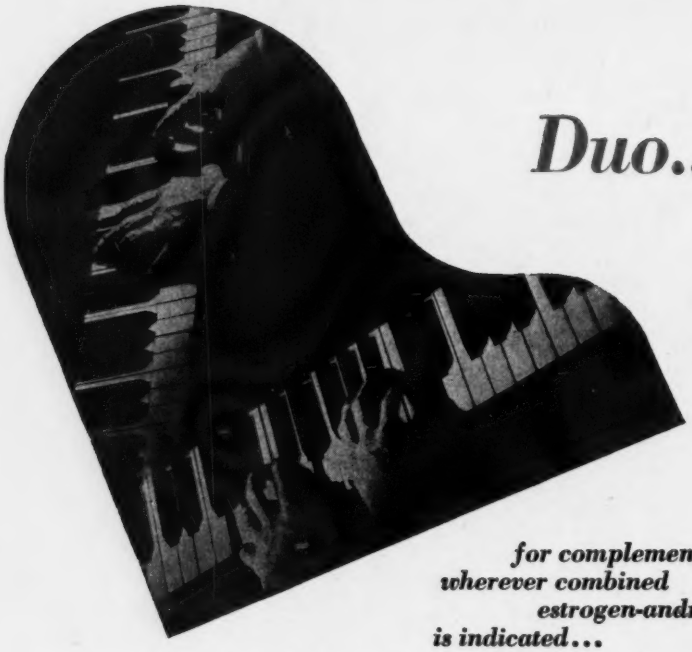

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ENDOCRINOLOGY

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THE COMPARATIVE VALUE OF CORTISONE, 17-HYDROXYCORTICOSTERONE AND ADRENAL CORTEX EXTRACT GIVEN BY CONTINUOUS INTRAVENOUS INJECTION IN SUSTAINING THE ABILITY OF THE ADRENALECTOMIZED RAT TO WORK

DWIGHT J. INGLE, JAMES E. NEZAMIS AND ERVING H. MORLEY

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

DURING the past 18 years one of us (D.J.I.) has used the work performance of the adrenalectomized rat as a criterion of the biologic potency of adrenal cortex extracts and steroids. The ability of the adrenalectomized-nephrectomized rat to work in response to faradic stimulation of the quadriceps muscle is the most severe test of the efficacy of replacement therapy with which we have had experience. In earlier studies (Ingle, 1940; Ingle and Kuizenga, 1945) it was not found possible to fully replace the secretion of the rat's own adrenal cortices by the administration of any one of the known biologically active adrenal steroids. However, these compounds were administered by intermittent injection, a procedure which does not simulate the continuous secretion of hormones by the gland and, moreover, the dose-response relationship was not fully explored. More recently, it was found that the continuous intravenous injection of large amounts of adrenal cortex extract sustained a normal output of work in adrenalectomized-nephrectomized rats over a 24 hour period (Ingle and Nezamis, 1949).

In the present experiment doses of cortisone, 17-hydroxycorticosterone and adrenal cortex extract which had been shown in earlier studies to elicit maximal work performance were each administered by continuous intravenous injection to adrenalectomized-nephrectomized rats. The peak work performance of rats treated with pure steroids remained inferior to that of non-adrenalectomized rats and of adrenalectomized rats treated with adrenal cortex extracts.

Received for publication August 3, 1951.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gms. The work test was carried out according to Ingle (1944). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the stimulation of muscle immediately following removal of both adrenal glands and both kidneys. The quadriceps muscle of the left hind leg was weighted with 100 gms. A Nerve Stimulator, Model B (Upjohn), was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 gram-centimeters of work. Stimulation was continued until muscular responsiveness was lost. The animals were enclosed in a cabinet with the temperature constant at $28 \pm 0.5°$ C.

The adrenal cortex extract was prepared from mixed hog and beef adrenal glands and contained the biologic activity equivalent of 0.1 mg. of 17-hydroxycorticosterone per cc. as assayed by the liver glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947). The adrenal cortex extract and the two steroids were each dissolved in water containing 5 per cent ethanol, 0.9 per cent sodium chloride and the potassium salt of penicillin G (Upjohn). The fluid load was 20 cc. per 24 hours per rat and the penicillin dose was 5000 units per 24 hours per rat. The injections were made into the jugular vein by means of a continuous injection machine which was incorporated into the fatigue apparatus. By means of this apparatus 12 rats can be studied simultaneously.

EXPERIMENTS AND RESULTS

Fifteen nephrectomized rats having their adrenal glands intact served as controls for 75 adrenalectomized-nephrectomized rats which were divided into equal groups according to treatment with the following amounts of hormone per 24 hours per rat: 20 cc. adrenal cortex extract; 2, 3 and 4 mg. of 17-hydroxycorticosterone; and 4 mg. of cortisone. The test substances were represented equally in each individual experiment. Each position on the 12-place fatigue apparatus and each of 12 automatic recorders was represented equally among the rats on each test substance. The average number of recorder revolutions per group together with the standard deviations of the average are summarized in Table 1.

TABLE 1. WORK PERFORMANCE OF ADRENALECTOMIZED-NEPHRECTOMIZED RATS GIVEN ADRENAL CORTICAL STEROIDS AND EXTRACT BY CONTINUOUS INTRAVENOUS INJECTION. TOTAL RECORDER REVOLUTIONS. AVERAGES AND STANDARD DEVIATIONS FOR 15 RATS PER GROUP

Experiment	
non-adrenalectomized; no hormone	$48,637 \pm 2,961$
Adrenalectomized; 20 cc./24 hours adrenal cortex extract	$46,292 \pm 2,289$
adrenalectomized; 2 mg./24 hours 17-hydroxycorticosterone	$37,691 \pm 1,950$
adrenalectomized; 3 mg./24 hours 17-hydroxycorticosterone	$38,519 \pm 1,999$
adrenalectomized; 4 mg./24 hours 17-hydroxycorticosterone	$40,040 \pm 1,710$
adrenalectomized; 4 mg./24 hours cortisone	$40,788 \pm 1,767$

The average performance of the non-adrenalectomized rats was a little greater than that of adrenalectomized rats treated with adrenal cortex extract but the difference may have been owing to chance. The average work output of each of these two groups was superior to that of each of the groups treated with the pure steroids and the reliability of the differences meets the usual requirements for statistical significance. When the results of the several groups treated with pure steroids are considered together the superiority of the control and extract treated animals would seem to be clearly established. The group differences in work output by rats treated with cortisone and several doses of 17-hydroxycorticosterone were not statistically significant but the possibility that true differences exist cannot be discounted.

DISCUSSION

The data of this experiment show that adrenal cortex extract elicits a greater peak response in the work test than does cortisone or 17-hydroxycorticosterone. The amount of each preparation required to elicit the peak response characteristic of that preparation was very large. Ingle and Nezamis (1949) found that 20 cc. of adrenal cortex extract per 24 hours per rat was required for optimal replacement therapy under similar experimental conditions. Ingle, Nezamis and Morley (1951) found that 4 mg. of cortisone per 24 hours per rat was required for a peak response to this steroid. Since 17-hydroxycorticosterone has greater biologic potency than cortisone per unit weight (Ingle and Kuizenga, 1945) we did not test doses larger than 4.0 mg. per rat per 24 hours in the present experiment. As assayed by the liver glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947), 20 cc. of adrenal cortex extract has the biologic activity equivalent of 2.0 mg. of 17-hydroxycorticosterone.

In this experiment we have eliminated one possible cause of differences in apparent efficacy of replacement therapy by administering the hormones by continuous intravenous injection.

The question as to whether any one of the known adrenal steroids can fully substitute for the natural secretion of the adrenal cortices has not been answered to the satisfaction of all of the investigators in the field. Conn, Louis and Fajans (1951), have suggested that 17-hydroxycorticosterone may possibly be the only natural secretory product of the adrenal cortices and have reviewed the supporting evidence. The evidence that this compound is secreted by the adrenal cortex is very strong. The principal lines of evidence that it is not the only biologically important secretory product of the adrenal cortex are: first, as shown in the present experiment, 17-hydroxycorticosterone does not fully replace the secretion of the animal's own adrenal cortices and, second, extracts of adrenal glands not

only elicit a greater peak response than does 17-hydroxycorticosterone but non-crystalline fractions of adrenal cortex extracts (the "amorphous fraction") have been prepared which have greater biologic activity per unit weight than does any known adrenal steroid (see Ingle, 1950 for review). The possibility that the "amorphous fraction" represents a mixture of known compounds and inactive impurities deserves consideration. However, its potency per unit weight in sustaining the life of adrenalectomized animals is much greater than that of 17-hydroxycorticosterone.

SUMMARY

Adrenalectomized-nephrectomized male rats having an initial weight of 200 ±2 gms. were anesthetized with barbiturates and subjected to faradic stimulation of the quadriceps muscle to lift a 100 gm. weight 5 times per second. Adrenal cortex extract and 17-hydroxycorticosterone and cortisone were each dissolved in 5 per cent ethanol and administered by continuous intravenous injection. The experiment was continued until muscular responsiveness was lost. Fifteen rats were studied at each of the following dosages: adrenal cortex extract, 20 cc. per 24 hours per rat; cortisone, 4 mg. per 24 hours per rat; and 17-hydroxycorticosterone, 2, 3 and 4 mg. per 24 hours per rat. These doses had been found in other experiments to sustain optimal work output for each respective preparation. Fifteen nephrectomized, non-adrenalectomized rats served as additional controls. The average total work done by non-adrenalectomized rats and adrenalectomized animals which received adrenal cortex extract was significantly greater than the total work of rats treated with optimal amounts of either cortisone or 17-hydroxycorticosterone.

Acknowledgment

We wish to express our appreciation to Dr. Augustus Gibson, The Medical Division of Merck and Company, who supplied the cortisone and to Dr. W. J. Haines, Endocrinology Department, The Upjohn Research Laboratories, who supplied the 17-hydroxycorticosterone for these studies.

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THE DEMONSTRATION OF A NON-ESTROGENIC UTERINE STIMULATING AND ESTROGEN AUGMENTING SUBSTANCE IN PREGNANT MARES' URINE[1]

HERMAN COHEN[2,3] AND ROBERT W. BATES

From the Department of Biology, New York University Graduate School of Arts and Sciences, New York, N. Y. and the Squibb Institute for Medical Research, New Brunswick, N. J.

INTRODUCTION

THE occurrence of substances, in the extracts of human urine and of testes, that are capable of augmenting the effects of estrogens or androgens has been postulated and demonstrated by various workers. Freud and co-workers (1933, 1935) obtained factors from extracts of testes and human urine that augmented the effects of androgens, although possessing no androgenic activity themselves. Emmens (1938) described the presence of substances in the phenolic fraction of normal human female urine which in themselves were non-estrogenic, but when given orally, increased the potency of estriol injected subcutaneously. Emmens (1939) also demonstrated that when normal human female urine was fractionated into phenolic, neutral, and acidic fractions and each fraction was assayed separately, the sum of the potency of the fractions was about 50 per cent less than that of the original starting material or that obtained when the fractions were recombined. He also reported that both the acidic and neutral fractions were capable of augmenting the effect of both estriol and estrone. Similarly, Pincus and Pearlman (1941) obtained data indicating that normal men and women excrete non-ketonic materials which enhance the activity of urinary estrogens. Smith *et al.* (1939) reported that pregnandiol and 2,7-dihydroxynapthalene enhanced the estrogenic effect of estrone on the vaginas of spayed rats, but the extent of the potentiation was not described.

Sheinerman and Gershtein (1947) have reported that injection of as-

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[2] Present Address: Squibb Institute for Medical Research, New Brunswick, N. J.

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corbic acid with estrone increases the uterine weight of immature mice about three times as much as does estrone alone, which observation we could not confirm. Schiller (1946) presented data showing that the estriol fraction obtained from pregnancy urine of humans was more active than similar amounts of crystalline estriol in causing withdrawal bleeding in the castrate monkey, and he attributed this to the presence of enhancing substances in the crude extract not present in the crystalline estriol. However, these two preparations were equally potent when tested by the vaginal cornification technique, using adult ovariectomized rats.

Our attention was focused on this subject by observations made during attempts to isolate non-ketonic estrogens from pregnant mares' urine. Fractionation of the non-ketonic materials into three fractions always resulted in a 50 per cent loss of biological activity without similar loss by chemical assay. This suggested the presence of an augmenting factor. One fraction was separated, which was non-phenolic, and, though not estrogenic in adult castrate female rats, would increase uterine weight in immature rats and potentiate the response to both natural estrogens and stilbestrol when measured by the vaginal smear technique.

METHODS AND MATERIALS

Commercially prepared estrogenic concentrates, phenolic fraction, from pregnant mares' urine* were used. These concentrates were prepared by the usual solvent fractionation methods and, presumably, were entirely phenolic in nature. These phenolic fractions were separated into ketonic and non-ketonic fractions by means of Girard's P reagent (Girard and Sandulesco, 1936). The non-ketonic fractions were subjected twice more to treatment with Girard's reagent in order to assure removal of all the ketonic estrogens. The non-ketonic fraction was then evaporated to dryness, dissolved in toluene and fractionated into a 0.1N NaOH soluble Fraction B, a 1N NaOH soluble Fraction C and a neutral Fraction D, as illustrated in Figure 1. The term "neutral" as used here is to be interpreted as meaning the apparently non-phenolic fraction remaining in toluene after extraction with 0.1N and 1N NaOH.

Bioassays were performed by a modified Kahnt-Doisy method utilizing adult ovariectomized white rats of a highly inbred strain developed in the Squibb Laboratories. As a standard, crystalline estrone was used at three dosage levels. Each extract was assayed at three dosage levels, 20 to 40 animals were used per dosage level. The extracts and estrone standard were dissolved in corn oil and subcutaneous injections given in two doses of 0.1 ml. each at 5 hour intervals. Forty-eight hours later vaginal smears were examined for signs of estrus. All potencies are expressed as International Units using estrone as a standard.

When bioassays were made with different fractions simultaneously administered into different parts of the body, the active estrogenic fraction was injected at three dosage levels but the amount of the inert fraction, such as Fraction D, was kept constant at all three dosage levels of the estrogen.

For studies on the effect upon uterine weight, subcutaneous injections were given

* Used in the preparation of Amniotin (Squibb).

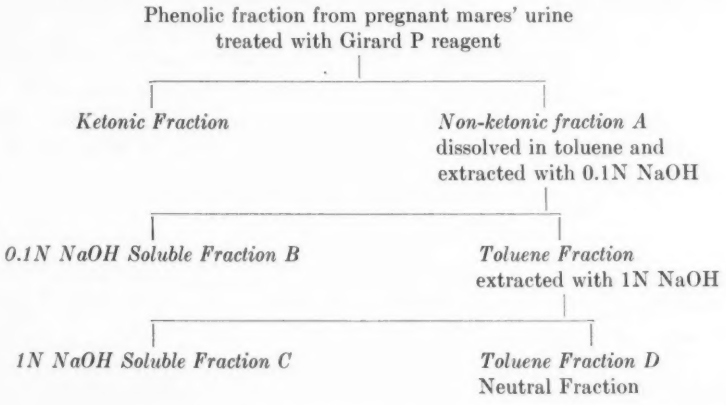
once daily in 0.2 ml. of corn oil, for three days. On the fourth day the vaginal smears were examined to determine the absence or presence of vaginal cornification. The animals were then sacrificed and the uteri excised and weighed on a torsion balance.

RESULTS

1) *Demonstration of Augmentation*

The non-ketonic fractions obtained from phenolic preparations 71509 and 70722 were fractionated according to the scheme in Figure 1. Each of the three fractions thus obtained was bioassayed. Five equal portions of preparation 71509 were fractionated at different times with similar results.

FIG. 1. Scheme of fractionation of the phenolic estrogen fraction.



The averaged data from the bioassays of these five experiments are presented in Table 1 along with data from a single fractionation of preparation 70722. These data demonstrate the presence of substances in pregnant mares' urine which are estrogenically inactive themselves, but are capable of augmenting the estrogenic activity of other fractions which are estrogenic. It can be seen that the 0.1N NaOH Fraction B contains 50 per cent of the total starting estrogenic activity while the 1N NaOH Fraction C and the neutral Fraction D contain only 3 to 10 per cent and 0.3 to 0.6 per cent, respectively. When Fractions B, C, and D were recombined in the proportion in which they occurred in the starting material A and were assayed, the full estrogenic potency obtained with the starting material A was recovered.

2) *Localization of Augmentor*

In which fraction or fractions was the augmentor to be found? From the data in Table I it was inferred that the augmenting factor or factors must

TABLE 1. TOTAL ESTROGENIC POTENCY OF THE THREE FRACTIONS OBTAINED FROM THE NON-KETONIC PHENOLIC EXTRACT OF PREGNANT MARES' URINE, AND OF A RECOMBINATION OF THESE FRACTIONS. STANDARD ERROR OF ASSAY WAS APPROXIMATELY ±13 PER CENT

	From #71509		From #70722	
	Potency		Potency	
	Total	Relative to A	Total	Relative to A
	I.U.	%	I.U.	%
(1) Starting Non-ketonic Fraction A	3,620,000	100.0	3,200,000	100.0
(2) 0.1N NaOH Fraction B	1,715,000	47.3	1,800,000	56.2
(3) 1N NaOH Fraction C	106,500	3.0	330,000	10.3
(4) Neutral Fraction D	9,000	0.3	20,000	0.6
(5) Sum of 2+3+4	1,828,000	50.5	2,140,000	67.0
(6) Recombined B+C+D	3,665,400	100.0	3,600,000	112.5

be located either in the 1N NaOH Fraction C or the neutral Fraction D, which fractions have little or no estrogenic activity. A portion of the non-ketonic extract A was fractionated as in Figure 1. Bioassays were carried out upon proportional combinations of the 0.1N NaOH Fraction B with the 1N NaOH Fraction C or with the neutral Fraction D. The results, Table 2, indicated that the potentiating factor or factors were mainly present in Fraction D inasmuch as with this fraction, which contained almost no estrogenic activity, the estrogenic activity of the 0.1N NaOH fraction was increased by 89 per cent. The small increase with Fraction C was not significant.

TABLE 2. DATA DEMONSTRATING THE PRESENCE OF THE AUGMENTOR IN NEUTRAL FRACTION D. THE ORIGINAL NON-KETONIC FRACTION HAD A TOTAL POTENCY OF 2,000,000 I.U.

Fraction	Total potency		Increase on combining
	Found	Calculated	Found/Calculated
	I.U.	I.U.	
Fraction B (0.1N NaOH)	975,000 ±17%	—	—
Fraction C (1N NaOH)	165,000 ±16%	—	—
Fraction B+C (combined)	1,380,000 ±16%	-1,140,000	1.14
Fraction B (0.1N NaOH)	975,000 ±17%	—	—
Fraction D (Neutral)	<5,000	—	—
Fraction B+D (combined)	1,850,000 ±16%	980,000	1.89

3) *Mode of Action of Augmenting Substance*

Is the action of the augmentor systemic, "hormonal," in nature or is it due to the presence of substances which act locally at the site of injection to delay the absorption of the active estrogens? The 0.1N NaOH Fraction B, which contained the urinary estrogens, was injected into castrate rats in the shoulder region and the augmentor (neutral Fraction D) was injected simultaneously into the hip area. The results obtained showed that

the augmentor was exerting its effect through a systemic mechanism inasmuch as potentiation was obtained when Fraction D was injected into the same (Table 2) or different sites (Figure 2) as Fraction B.

In an attempt to find a quantitative method of assay for the augmentor, increasing amounts of Fraction D were injected simultaneously with Fraction B but on opposite sides of the body. The results, shown in Figure 2, indicate that the potentiation increases in a regular manner with increasing

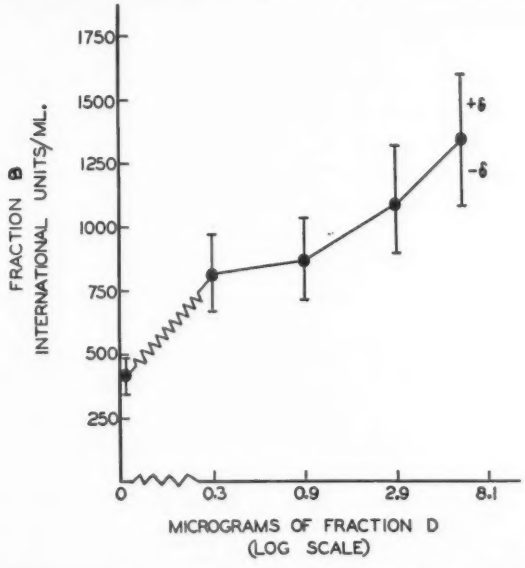


FIG. 2. Biological response expressed in International Units, using estrone as a standard, produced by the 0.1N NaOH Fraction B when injected in conjunction with the augmentor Fraction D. Each fraction injected subcutaneously into different sites in the animal.

amounts of the augmentor. Statistical analysis showed that all responses were significantly increased by even the smallest amount of Fraction D (0.3 microgram) but the response with 6.6 micrograms was not significantly different from that with 0.3 microgram.

Further evidence of the systemic nature of the augmentor action is presented in Table 3 where data showing the oral effectiveness of the augmentor are presented. When given orally the augmentor appeared to be as active as when given subcutaneously.

4) *Combination of Fraction D with Various Estrogens*

The potentiating effect of Fraction D upon various other estrogens, crystalline estrone, diethylstilbestrol, and partially purified phenolic frac-

TABLE 3. DATA DEMONSTRATING THE ORAL EFFECTIVENESS OF THE AUGMENTOR IN POTENTIATING THE EFFECT OF ADMINISTERED ESTROGENS. THE INDICATED AMOUNT OF AUGMENTOR WAS GIVEN TO ALL RATS WITHIN AN ASSAY GROUP WHILE THREE DOSAGE LEVELS OF FRACTION B WERE USED

Fraction	Route of administration	Estrogenic activity
Augmentor Fraction D 5.6 μg.	Oral	I.U./ml. None
Augmentor Fraction D 28 μg.	Oral	None
0.1N NaOH Fraction B	Subcutaneous	1780 ± 13%
0.1N NaOH Fraction B plus Augmentor Fraction D 28 μg.	Subcutaneous Subcutaneous	3413 ± 15%
0.1N NaOH Fraction B plus Augmentor Fraction D 28 μg.	Subcutaneous Oral	3443 ± 16%
0.1N NaOH Fraction B plus Augmentor Fraction D 5.6 μg.	Subcutaneous Oral	3634 ± 16%

tions of pregnant mares' urine, was studied and the results presented in Table 4. It can be seen that with all the preparations tested except crystalline estrone the potentiating factor is capable of increasing the activity of the subcutaneously administered estrogen by approximately 100 per cent. Estrone is the exception, the increase being only 50 per cent.

Fraction D is also effective in potentiating the effect of estrogens on the vaginal mucosa of ovariectomized mice, the degree of potentiation upon injection of Fraction B in conjunction with Fraction D into mice being similar to that found in rats (Rakoff, 1950).

TABLE 4. EFFECT OF AUGMENTOR UPON THE ESTROGENIC POTENCY OF CRUDE, CRYSTALLINE AND SYNTHETIC ESTROGENS

Estrogen	Fraction D* dose	ED-50‡	Increase
	μg.		%
Estrone	0	1.65 μg.	
Estrone	10	1.10 μg.	50
Stilbestrol	0	0.315 μg.	
Stilbestrol	5	0.143 μg.	120
Amniotin†	0	17 I.U.	
Amniotin	5	10 I.U.	70
Fraction B	0	14 I.U.	
Fraction B	5	7 I.U.	100

* 224 μg. of Fraction D fails to cause estrus.
† SQUIBB preparation of phenolic fraction of pregnant mares' urine.
‡ Dosage which produces cornification of the vagina in 50 per cent of the rats.

5) *Effect of Fraction D upon the Uterus of the Immature Rat*

Does the augmentor have a direct action on the uterus although it does not cornify the vaginal mucosa? Intact, ovariectomized, adrenalectomized, and ovariectomized-adrenalectomized 21 day old immature females weighing 55–60 grams were used. The animals were injected daily with 28 micrograms of Fraction D for three days. On the fourth day the animals were sacrificed and the uteri excised and weighed. The results are presented in Table 5. These data show that Fraction D is capable of doubling the uterine weight of the injected animals, a response which is striking in view of the fact that there were no estrogens present in this fraction as determined by injections of 56 micrograms of Fraction D daily into 40 adult ovariectomized rats for a period of four days. Daily vaginal smears showed no signs of estrus.

TABLE 5. EFFECT UPON UTERINE WEIGHT OF AUGMENTOR FRACTION D WHEN INJECTED ONCE DAILY SUBCUTANEOUSLY FOR THREE DAYS INTO RATS

Type rat	No. rats	Total dose fraction D	Body wt.	Uterine wt.	Vaginal orifice	Vaginal smear
		μg.	gm.	mg.		
Uninjected Controls	10	—	63	24.6 ± 0.8	Closed	Diestrus
Intact	10	84	65	44.0 ± 4.1	Closed	Diestrus
Ovariectomized	7	84	63	42.4 ± 4.7	Closed	Diestrus
Ovariectomized-Adrenalectomized	13	84	61	44.9 ± 3.6	Closed	Diestrus
Hypophysectomized	5	—	111	56.4 ± 7.4	Open	Diestrus
Hypophysectomized	7	112	114	121.0 ± 14.1	Open	Diestrus

Histological examination of the uteri stimulated by Fraction D revealed a columnar epithelium which showed no consistent increase in height, but as many as six mitotic figures per section were evident. The endometrium showed a dispersal of the stromal nuclei as a result of which less accumulation was seen directly under the epithelium. The nuclear types were not changed but mitotic figures as well as occasional lymphocytes were observed in the stroma. The histology of the organ generally indicated a situation suggestive of mild estrogenic stimulation.

The doubling of uterine weight by Fraction D was also obtained in hypophysectomized animals. This indicates a lack of hypophyseal mediation in the action of Fraction D upon the uterus (Table 5).

Further indication of a distinct difference between the effect of estrone on the uterus and that of Fraction D upon the uterus is demonstrated by the data in Table 6. From these data it may be seen that similar uterine weights are produced by 16 micrograms of estrone administered over a period of four days and by 112 micrograms of Fraction D administered over

a similar period. However, whereas the estrone administration produced cornification and premature opening of the vagina, no such situation prevailed in the animals treated with Fraction D.

TABLE 6. DIFFERENCE IN RESPONSE OF 21 DAY OLD IMMATURE OVARIECTOMIZED FEMALE RATS TO OIL SOLUTIONS OF ESTRONE AND FRACTION D. ANIMALS INJECTED ONCE DAILY FOR FOUR DAYS AND SACRIFICED ON FIFTH DAY

Treatment (total dose)	No. rats	Body wt.	Uterine wt.	Vaginal orifice	State of estrus
		gm.	mg.		
Estrone 16 μg.	5	52	53 ± 2	Open	Estrus
Fraction D 112 μg.	7	67	53 ± 6	Closed	Diestrus
Controls	5	66	26 ± 0.6	Closed	Diestrus

6) *Effect of Estrone plus Fraction D upon the Uterus of the Immature Rat*

Does Fraction D augment the effect of estrone upon the uterus? Varying doses of estrone were injected daily for three days into two groups of 21 day old immature rats. One group received estrone injections only, and the other group received estrone plus a constant dose of Fraction D. The results are presented in Figure 3, and at the 6 and 12 μg. level indicate some potentiation by Fraction D of the effect of estrone on the immature rat uterus.

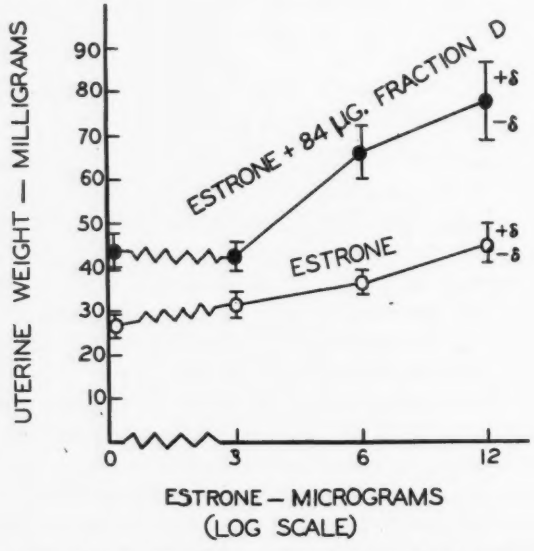


FIG. 3. Effect upon the uterine weight of immature rats of variable amounts of estrone with and without 84 micrograms of Fraction D. Total dosage for 3 days is shown. Rats were autopsied on fourth day. Six rats per group.

7) *Effect of Fraction D on Oviducal Weight in Chicks*

Starting on the third day after hatching, estrone, Fraction D, and a mixture of the two were injected into groups of White Leghorn baby chicks (Table 7). In order to obtain a significant oviducal weight increase, larger doses were required than in rats. Fraction D was approximately one-half as potent as estrone, as measured by weight increases of the oviduct.

TABLE 7. EFFECT OF AUGMENTOR, FRACTION D, AND ESTRONE ON THE WEIGHT OF THE OVIDUCT OF BABY CHICKS. SUBCUTANEOUS INJECTIONS WERE GIVEN ONCE DAILY FOR FOUR DAYS. CHICKS WERE AUTOPSIED ON THE FIFTH DAY

	Total dose	No. chicks	Body wt. average	Oviduct wt.
	μg.		gm.	mg.
Uninjected Controls	—	5	52	4.2 ± 0.2
Estrone	200	3	59	10.2 ± 1.4
Estrone	1000	4	59	27.8 ± 3.7
Fraction D	216	4	54	5.9 ± 0.5
Fraction D	432	4	53	9.3 ± 0.5
Estrone + Fraction D	200 + 216	4	49	19.1 ± 3.5

The one series in which estrone was injected together with Fraction D indicated potentiation of effectiveness of estrone in causing an increased oviducal weight.

DISCUSSION

The data presented in this study demonstrate the presence in pregnant mares' urine of substances which, although estrogenically inactive by themselves, are capable of potentiating the biological effect of estrogens upon the vaginal mucosa of the adult spayed rat. Such substances have been previously described in human urines by Emmens (1939) andPincus and Pearlman (1941). In pregnant mares' urine the potentiator was found in the neutral fraction whereas Emmens has reported that in human urine such potentiating factors are present in both the neutral and the acidic fractions. Fraction D, besides augmenting the effect of estrogens on the vaginal mucosa, has also been found capable of causing an increased uterine weight in immature rats, an effect which has not been described by the investigators working with human urine.

Working with androgens Freud *et al.* (1933, 1935) found delayed absorption to be the mechanism by which certain fractions from testicular extracts potentiated the effect of androgens. In the experiments above, the potentiation by Fraction D of the action of estrogens upon the vaginal mucosa does not seem to be due to a delayed absorption from the site of injection because Fraction D is as effective when given orally or by injection into different sites in the animal as when administered together with estrogens.

The following negative results indicate that the physiological role of Fraction D may be definitely limited to certain aspects of estrogenic activity. Fraction D was found to have no effect on the normal estrus cycle of mature intact female rats injected daily for a period of one week. Hence it does not seem to potentiate or inhibit the normal estrogen inactivating systems of the organism. Also, Leathem (1951) found that Fraction D did not modify the action of androgens on the seminal vesicles or prostates of immature male rats nor did it modify the effect of estrogens on the seminal vesicles of such animals.

The effects of Fraction D on the uterus of immature rats seems to be a direct effect and not a potentiation of the effect of endogenous estrogen, because Fraction D is effective in ovariectomized, ovariectomized-adrenalectomized, as well as intact immature rats, thus ruling out all the known sources of endogenous estrogens.

Estrone was found to be twice as effective as Fraction D in increasing the oviduct weight in baby chicks whereas it was ten times more active than Fraction D in increasing the weight of the rat uterus. There was an indication of a potentiating effect of Fraction D on the effect of estrone on the weight of the chick oviduct and the rat uterus.

In contrast to the similarity in potency of estrone and Fraction D on uterine and oviduct weight tests, estrone was found to be more than 224 times more effective than Fraction D in causing cornification of the vagina when given alone. When injected simultaneously, an equal weight or even less of Fraction D relative to the estrogen would nearly double the effectiveness of estrone and other estrogens. This dichotomy of action of Fraction D on the uterine weight and vaginal cornification is in contrast to the action of the usual estrogens, and indicates that Fraction D contains a substance or substances with different physiological properties than those usually ascribed to estrogens.

SUMMARY

The phenolic, non-ketonic fraction from pregnant mares' urine was re-extracted from toluene with aqueous sodium hydroxide. The "neutral" fraction remaining in the toluene, though unable to cause vaginal cornification alone, when given orally or by injection was found to potentiate the estrogenic potency of estrone, diethylstilbestrol or the estrogenic fraction from mares' urine when administered simultaneously but at different sites.

This "neutral" fraction was found to increase the uterine weight in hypophysectomized, ovariectomized or adrenalectomized rats. It also increased the oviducal weight in baby chicks.

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THE RELATION OF SOME STEROID HORMONES TO β-GLUCURONIDASE ACTIVITY[1]

ERNEST KNOBIL[2]

From the Department of Zoology, Cornell University, Ithaca, New York

RENEWED interest in the physiological function of the enzyme β-glucuronidase was initiated by the report of Fishman (1940) that the feeding of glucuronidogenic substances to mice and dogs effected an increase in the β-glucuronidase activity of liver, kidney and spleen, while the enzyme concentration of the sex organs remained unchanged. However, the administration of estrogens to ovariectomized mice caused an increase in β-glucuronidase only in the uterus and possibly the vagina and conversely, ovariectomy resulted in a marked decrease in uterine β-glucuronidase activity (Fishman and Fishman, 1944; Fishman, 1947). It was concluded from these observations that β-glucuronidase catalyzes the synthesis of glucuronides *in vivo* and that the observed increases in enzyme activity represented an enzyme adaptation phenomenon in response to the presence of increased substrate. Fishman (1947) further postulated that β-glucuronidase plays a fundamental role in the physiological action of estrogenic hormones and that this role is concerned with the "metabolic conjugation" of estrogens with glucuronic acid prior to the utilization of the complex by the tissue.

Karunairatnam and Levvy (1949) and Karunairatnam, Kerr and Levvy (1949) on the other hand, presented convincing evidence that the enzyme system responsible for glucuronic acid conjugation is separate and distinct from β-glucuronidase. It was noted (Levvy, Kerr and Campbell, 1948) that liver regeneration following injury by various means was accompanied by a rise in β-glucuronidase activity. On the basis of these and other observations, Levvy *et al.*, put forward the hypothesis that the increase in the β-glucuronidase concentration of a tissue is merely an index of cell proliferation and tissue growth regardless of the nature of the stimulus.

In view of the fact that a relationship between uterine β-glucuronidase activity and estrogen levels has been clearly demonstrated, it seemed of fundamental importance to determine whether changes in β-glucuronidase activity indeed represent generalized alterations in growth processes or

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[2] Fellow of the Schering Corporation. Present address: Harvard School of Dental Medicine, Boston 15, Massachusetts.

whether such changes specifically reflect the morphological and physiological processes governed by the estrogenic hormones. It was thought that an investigation of β-glucuronidase activity in the accessory organs of reproduction under a variety of hormonal environments might prove fruitful in this regard. The present report deals with studies of the β-glucuronidase activity of the uterus, mammary gland, prostate gland and seminal vesicle in relation to the action of estrogens and other steroid hormones.

MATERIALS AND METHODS

Animals and preparation of tissues. Rats of the Long-Evans strain and rabbits of undetermined breeding were used in these experiments. The animals of any one experimental group were selected for comparable age and weight, and, in the case of immature female rats, litter mates were employed whenever possible. All operations were performed by standard procedures under ether anesthesia. The hormones used in this study were estradiol benzoate, testosterone propionate, desoxycorticosterone acetate, and progesterone.[3] These were administered in oil.

At the termination of an experiment, the animals were anesthetized with ether and bled from the carotids. The tissue to be assayed was quickly removed, trimmed of adhering fat and connective tissue, and placed in a chilled, physiological saline solution. The tissue was then blotted dry on paper toweling, and quickly weighed on a torsion balance. A weighed portion of tissue was dried to constant weight at 90–100° C. for the determination of water content and the remainder assayed. In the case of the uterus and the seminal vesicle, the fluid, when present, was expelled prior to weighing. β-glucuronidase activity was determined by the method of Talalay, Fishman and Huggins (1946). The substrate, phenolphthalein mono-β-glucuronide was prepared by the method of Fishman, Springer and Brunetti (1948). The unit of β-glucuronidase activity is defined as that amount of enzyme which will liberate one gamma of phenolphthalein in one hour at 38° C. from phenolphthalein glucuronide at pH 4.5 under standard conditions.

Histological and histochemical procedures. The method of Friedenwald and Becker (1948) for the histochemical localization of β-glucuronidase was utilized. The incubation time varied with the tissue under study (12 hours for rat uterus; 48 hours for mammary gland). Counterstaining was omitted in most cases. Standard procedures for the preparation and staining of whole mounts of mammary gland and other tissues were followed.

EXPERIMENTAL

β-glucuronidase in the seminal vesicle and prostate. If the decrease in uterine β-glucuronidase concentration following ovariectomy can be explained in terms of a cessation of mitotic activity with a general decrease in cellular metabolism and consequent involution of the tissue, then the atrophy of the seminal vesicle and prostate glands following castration in the male should also result in a similar decrease in enzyme activity.

Adult male rats were castrated and their seminal vesicles and prostate glands assayed for enzyme activity approximately 40 days after the opera-

[3] The hormones used in this study were generously supplied by the Schering Corporation, Bloomfield, New Jersey.

tion. Tissues from control animals were assayed simultaneously. The prostate glands removed from the castrated did not afford sufficient tissue for individual enzyme determinations, and glands from at least two animals had to be pooled. Control experiments showed that prostatic fluid did not contain measurable amounts of β-glucuronidase.

The drastic atrophy of both seminal vesicle and prostate consequent to the withdrawal of the male hormone was not paralleled by a decreased β-glucuronidase activity. The differences in enzyme values reported in Table 1 are not statistically significant.

TABLE 1. EFFECT OF CASTRATION ON β-GLUCURONIDASE LEVELS IN SEMINAL VESICLE AND PROSTATE GLANDS

Experiment	Number of rats	Body weight gm.	Organ weight mg.	Units β-glucuronidase per mg. dry wt.
Seminal vesicle				
Normal	6	309	378	13.7 ± 1.1[1]
Castrate	12	306	102	11.7 ± 0.9
Prostate				
Normal	14	307	280	33.2 ± 2.9
Castrate	24	335	27	31.7 ± 2.9

[1] Results in this and subsequent tables are given as means ± standard error of the mean or as mean and range.

There is excellent evidence that estrogens stimulate growth of the fibromuscular stroma in the male accessory glands and to a lesser extent that of the secretory epithelium, especially in the seminal vesicle (Overholser and Nelson, 1935; Tislowitz, 1939). Thus it was of interest to determine whether the effects of estrogen on the seminal vesicle and prostate glands of castrated males would be accompanied by a rise in β-glucuronidase activity.

Adult male rats, castrated for 40 days, were given daily subcutaneous injections of 15 gamma estradiol benzoate for 19 days. At the end of this period, prostates and seminal vesicles were assayed for enzyme content, some tissues being removed for histological study. Untreated, 40-day castrated males served as controls. The results of this experiment are summarized in Table 2.

Although growth of the prostates and seminal vesicles was not discernible by their weights, the β-glucuronidase activity of seminal vesicles removed from treated males was 100% greater than that found in the untreated, castrated animals. A less striking but distinct rise in enzyme concentration was also noted in the prostate glands. Histological examination of these tissues revealed that estrogen treatment had caused an increase in fibromuscular mass of the seminal vesicles, but no such change was noted in prostates from animals similarly treated. The secretory epithelium

TABLE 2. β-GLUCURONIDASE ACTIVITY IN PROSTATES AND SEMINAL VESICLES OF CASTRATED MALES TREATED WITH ESTRADIOL BENZOATE

Experiment	Number of rats	Body weight gm.	Seminal vesicle		Prostate	
			Weight mg.	Activity*	Weight mg.	Activity
Controls	10	334	93	9.5 (6.4–13.3)	43	22.9 (20.4–26.3)
Estrogen treated	12	273	103	19.0 (15.1–22.4)	36	33.8 (28.2–39.8)

* Units of enzyme per mg. dry wt.

of both glands appeared to be slightly higher and in a more active state when compared with the control tissues, although this difference was not striking.

Effect of testosterone on uterine β-glucuronidase activity. In the next experiment it was desired to determine whether the morphological and physiological changes induced by testosterone in the uteri of ovariectomized rats (Greep and Jones, 1950) would be accompanied by an increased concentration of uterine β-glucuronidase. Adult female rats, ovariectomized for 90 days, were given daily injections of one mg. testosterone propionate for 22 days. The controls consisted of untreated, ovariectomized females of the same age. The resulting data are presented in Table 3.

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON UTERINE β-GLUCURONIDASE ACTIVITY IN THE OVARIECTOMIZED RAT

Experiment	Number of rats	Body weight gm.	Uterine weight mg.	Units β-glucuronidase per mg. dry wt.
Controls	5	244	63.2 (49.6– 88.7)	9.6 (7.3–14.0)
Testosterone treated	6	264	295.0 (233.4–331.8)	22.8 (19.8–25.9)

Testosterone treatment effected a marked increase in uterine weight which was accompanied by a distinct rise in β-glucuronidase activity.[4]

Progesterone and uterine β-glucuronidase. The morphological changes produced in the uterus by progesterone and its interactions with estrogens with respect to this organ offered an excellent opportunity to follow the changes in uterine β-glucuronidase activity under a wide variety of conditions. The following experiments were performed in an attempt to further

[4] Desoxycorticosterone acetate administration to ovariectomized rats failed to effect uterine growth or a rise in uterine β-glucuronidase activity. Similarly, adrenalectomy in ovariectomized adult rats was without effect on uterine weight and uterine enzyme concentration.

clarify the relationship between β-glucuronidase activity and tissue growth in relation to these two hormones.

Segments of the uteri were removed from three rabbits within ten hours after copulation. These were assayed for β-glucuronidase activity, and samples were saved for histological examination. The animals were autopsied five days later, and at this time samples of uterine tissue from the opposite horn were removed for enzyme assay and microscopic study. In this manner, each animal served as its own control. Three other rabbits were ovariectomized for 20 days and their uteri studied as before.

Uterine segments removed from the rabbits in heat had an average β-glucuronidase concentration of 2.58 units per mg. dry weight. Uterine segments from these same animals five days after the induction of pregnancy average 2.35 enzyme units. It is seen that, in spite of the intense progestational proliferation of the endometrium observed in early pregnancy, the enzyme concentration did not exceed that found in estrus. On the other hand, the uterine β-glucuronidase level observed in rabbits ovariectomized for 20 days dropped to 0.89 units per mg. dry weight. It should be noted that the uterine β-glucuronidase activity of ovariectomized rats is greater than that of rabbits in heat.

To further study the relation of progesterone to changes in uterine β-glucuronidase activity, an investigation of the pseudopregnant rat was undertaken. The pseudopregnant rat also furnished ideal material for the testing of Levvy's "growth hypothesis" for changes in β-glucuronidase activity, since rapid and extensive tumor-like endometrial proliferations, the deciduomata, can be induced in one uterine horn while the contralateral horn remains unchanged. Under these conditions, both the deciduomata and the uninvolved uterine tissue are in the same hormonal environment.

Female rats were made pseudopregnant by electrical stimulation of the cervix and deciduomata were induced in one uterine horn by passing threads through the lumen 6 days later, the other horn in the same animal remaining as the control. On the ninth day of pseudopregnancy, the deciduomata and the opposite uterine horn were assayed for β-glucuronidase

TABLE 4. UTERINE β-GLUCURONIDASE IN PSEUDOPREGNANT RATS

Experiment	Number of rats	Body weight gm.	Weight of horn assayed mg.	Units β-glucuronidase per mg. dry wt.
Normal rat—right horn	8	204	116	46.1 ± 3.5
Pseudopregnant horn—* no deciduomata	10	210	84	35.8 ± 2.4
Pseudopregnant horn—deciduomata	10	210	953	53.0 ± 4.7

* 9th day of pseudopregnancy.

TABLE 5. THE EFFECT OF ESTROGEN AND PROGESTERONE ON THE UTERINE β-GLUCURONIDASE ACTIVITY IN THE OVARIECTOMIZED RAT

Experiment	Number of rats	Body weight gm.	Uterine weight mg.	Units β-glucuronidase per mg. dry wt.
No treatment	8	254	61.5 ± 2.3	9.3 ± 0.5
Progesterone (10 mg.)	4	231	50.7 ± 6.2	9.3 ± 0.8
Estradiol benzoate (2 gamma)	8	238	141.6 ± 12.6	26.8 ± 1.9
Progesterone (4 mg.) Estradiol benzoate (2 gamma)	8	236	88.4 ± 6.8	24.2 ± 2.0

activity, and the resulting values compared with each other and with those obtained from intact, control animals. The results are summarized in Table 4.

The data indicate that on the ninth day of pseudopregnancy the β-glucuronidase concentration of uterine horns devoid of deciduomata was significantly lower ($P < .05$) than that found in the corresponding uterine horns of normal control animals. Despite the intense proliferation of the decidual tissue, the enzyme concentration of the deciduomata did not significantly exceed that of the normal uterus ($P > .20$). The β-glucuronidase activity of decidual tissue, however, did exceed that of the control pseudopregnant horn.

The decrease in uterine β-glucuronidase observed on the ninth day of pseudopregnancy may be a reflection of lowered estrogen levels or an indication that progesterone inhibited the effectiveness of estrogens circulatting at normal levels (Leonard, Hisaw and Fevold, 1932). That the first possibility is probably the correct one is shown by the following experiment

Female rats, ovariectomized for approximately 90 days, were given one mg. of progesterone subcutaneously. The next day, one mg. progesterone plus 2 gamma of estradiol benzoate were administered by the same route. One mg. of progesterone alone was given on each of the two days following, and the enzyme assays performed on the fifth day of the experiment (72 hours after the estradiol injection). Another group of ovariectomized animals were injected subcutaneously with 2 gamma estradiol benzoate and their uteri assayed for β-glucuronidase activity 72 hours later. Females ovariectomized for 90 days but receiving no treatment served as one control, while others, similarly operated but receiving daily injections of one mg. progesterone for ten days, served as the other. The results of these experiments are shown in Table 5.

The data clearly show that although progesterone markedly inhibited the growth effects of estradiol on the uterus, it failed to depress the rise in the β-glucuronidase concentration which was induced by estradiol. Progesterone alone had no effect.

Uterine β-glucuronidase in the immature female rat. Kerr, Campbell and

Levvy (1949) noted that the β-glucuronidase concentration of the uterus, liver and kidney of immature mice exceeded that found in corresponding tissues removed from adult animals. The high figure for uterine β-glucuronidase in infant mice was taken as support for the view that in this organ, as in others, the activity of the enzyme is a measure of general growth processes. Since the bulk of the evidence presented in this report does not support this concept, a study of uterine β-glucuronidase in the immature female rat in relation to ovariectomy and estrogen administration seemed indicated.

The uterine β-glucuronidase concentration of immature female rats was determined under the following experimental conditions:

1. Intact immature females receiving no treatment (these and subsequent animals were sacrificed at 29 days of age).
2. Immature rats ovariectomized seven days prior to assay; no treatment.
3. Immature rats ovariectomized 17 days before enzyme assay; no treatment.
4. Immature females were ovariectomized at 21 days of age. Five days after the operation they were given 10 gamma estradiol benzoate subcutaneously and their uteri assayed for enzyme activity 72 hours after the hormone injection.

It may be seen from the data in Table 6 that deprivation of the ovaries for 17 days was without effect on uterine β-glucuronidase activity, but there was a significant decrease in uterine weight ($P < .01$). The administration of 10 gamma estradiol benzoate to immature females ovariectomized for 5 days resulted in a 156% increase in uterine weight. *This marked uterine growth was not accompanied by a rise in enzyme activity.*

TABLE 6. UTERINE β-GLUCURONIDASE ACTIVITY IN THE IMMATURE FEMALE RAT

Experiment[1]	Number of rats	Body weight gm.	Uterine weight mg.	Units β-glucuronidase per mg. dry wt.
Intact—no treatment	8	65	35.3 ± 1.3	27.0 ± 1.3
Ovariectomized 7 days. no treat.	4	62	39.5 ± 2.1	26.2 ± 2.5
Ovariectomized 17 days. no treat.	9	66	30.0 ± 1.2	27.2 ± 0.6
10 gamma estradiol benzoate[2] 5 days after ovar.	4	65	90.0 ± 4.4	28.5 ± 1.6

[1] All animals assayed at 29 days of age.
[2] Assayed 72 hours after the estradiol benzoate injection

The relation of the pituitary to mammary and uterine β-glucuronidase. The mammary gland of the rat seemed a favorable tissue for the study of β-glucuronidase and its possible relation to growth, since this organ prolifer-

ates rapidly when estrogens are administered, but fails to do so in the absence of the pituitary (Reece and Leonard, 1941).

Female rats were ovariectomized and hypophysectomized 4 days later. Some of these were given 10 gamma estradiol benzoate per day for 9 days starting the day after hypophysectomy. The remainder served as controls. Further controls consisted of ovariectomized rats treated with 10 gamma estradiol benzoate per day for 15 days (an average, 11 to 19 days) starting the day after the operation and intact, untreated adult females.

At autopsy, the inguinal mammary glands of the above animals were removed. The glands from one side were assayed for β-glucuronidase activity and those from the other prepared for histological study. The uteri of the hypophysectomized group were also assayed for enzyme content.

The data in Table 7 show that the administration of estrogen to rats ovariectomized the day before the initiation of hormone treatment resulted in a more than three fold increase in mammary β-glucuronidase activity when compared with intact, control animals. When estradiol benzoate was given to ovariectomized-hypophysectomized animals, however, a much smaller rise in the enzyme level of their mammary glands was obtained.

TABLE 7. β-GLUCURONIDASE ACTIVITY IN THE MAMMARY GLAND OF THE FEMALE RAT

Experiment	Number of rats	Body weight gm.	Units β-glucuronidase per mg. wet weight
Intact controls	4	207	0.35 (0.29–0.40)
Ovariectomized, 10 gamma estradiol benzoate/day for 15 days	8	182	1.34 (0.67–2.40)
Ovariectomized-hypophysectomized control	3	143	0.38 (0.32–0.45)
Ovariectomized-hypophysectomized, 10 gamma estradiol benzoate/day for 9 days	5	123	0.54 (0.34–0.68)

Attention should be called to the fact that the enzyme values reported in Table 7 in all probability do not quantitatively correspond to the actual β-glucuronidase concentration present in the glandular elements of the tissues under consideration, since a large part of the samples used was comprised of presumably inactive fat and connective tissues.

Comparison of mammary glands removed from the ovariectomized animals treated with estrogen for 15 days with intact controls revealed extensive development of the duct system with a marked increase in the number of end buds. In some cases alveoli appeared to be present, some of these being distended with fluid. The only histologic change noted, following

estrogen administration to ovariectomized-hypophysectomized females, however, was a possible slight increase in the diameter of the mammary ducts. The increase in β-glucuronidase activity obtained in this group is thought to be insignificant, since the mammary glands were nearly devoid of fat as a result of the toxic effects of estrogens on hypophysectomized animals, and the glandular tissue proper predominated in the sample. This was not the case in the hypophysectoimzed controls.

In contrast to the behavior of mammary glands from ovariectomized-hypophysectomized animals, the uteri of these rats responded to hormone treatment in terms of tissue growth as well as β-glucuronidase activity. Ovariectomized-hypophysectomized controls: 23.1 (18.5–26.8) units; Estrogen treated ovariectomized-hypophysectomized animals: 34.5 (28.1–40.3) units).

An attempt was made to localize histochemically β-glucuronidase in some of the mammary glands obtained from the above experiments. Unfortunately this was not successful. The reason for this failure probably lies in the fact that the enzyme concentration in these tissues was too low for the sensitivity of the method.

Some histological and histochemical considerations. The histochemical technique of Friedenwald and Becker (1948) for the histochemical localization of β-glucuronidase has not been too rewarding in this laboratory. Despite numerous attempts and close adherence to the original method, only general localization of the site of enzyme activity could be achieved. The results which were obtained do not allow detailed description of the distribution of glucuronidase at the cellular level as reported by Campbell and Levvy (1950) for the liver. The observations of Friedenwald and Becker, that β-glucuronidase appears to be concentrated in the region of the glandular epithelium of the endometrium of the rat, have been confirmed. Scattered enzyme activity could be detected in the remainder of the endometrium, but the myometrium failed to show any evidence of activity. The same general picture was obtained in the deciduomata. A good indication of the sensitivity of the histochemical method is that β-glucuronidase activity could not definitely be detected in the uteri of rats ovariectomized for 30 days.

All the observations on uterine β-glucuronidase activity reported to date for the mouse and the rat are based, of necessity, on the chemical assay of the whole uterus, i.e., endometrium and myometrium. Since the ratio of myometrium to endometrium was found to vary considerably with the hormonal environment of the uterus (see Table 8) and since β-glucuronidase appears to be principally confined to the endometrium, the following possibility was viewed with apprehension. The observed difference in enzyme activity between the uteri of ovariectomized immature and normal

adult rats may not represent actual changes in uterine enzyme concentration, but could reflect variations in the relative volumes of active endometrium and the other components of the uterus which appear to be low in β-glucuronidase activity.

In an attempt to resolve this problem, uteri of ovariectomized adults, normal adults and immature animals were sectioned transversely, stained, and the relative cross sectional areas of endometrium and myometrium measured with a polar planimeter. The ratio of the cross sectional area of the uterus to the area of the endometrium was then calculated in each instance. Assuming that (1) uterine β-glucuronidase activity is limited to the endometrium, (2) that the weight of one unit volume of endometrium approximately equals the weight of one unit volume of myometrium, and (3) that the water content of both is the same, an approximation of the units of β-glucuronidase activity per mg. dry weight of endometrium could be computed. Thus, if the enzyme concentration of a given uterine segment weighing 2 mg. is 80 units and the endometrium comprises one half the cross sectional area (or volume) of the segment, then 1 mg. of endometrium represents 80 enzyme units. The resulting values calculated for the three types of rat uteri are given in Table 8.

TABLE 8. CALCULATED ENDOMETRIAL β-GLUCURONIDASE ACTIVITY IN THE NORMAL ADULT, OVARIECTOMIZED ADULT AND NORMAL IMMATURE FEMALE RAT

Experimental animal[1]	Ratio $\frac{\text{Total area}}{\text{Area of endometrium}}$	Units β-glucuronidase per mg. dry weight	
		Whole uterus	Endometrium
Normal adult	2.1	40[2]	84
Ovariectomized adult	3.4	12	41
Normal immature	2.4	27	65

[1] Three animals in each group.
[2] Enzyme values based on a large number of previous experiments.

On the basis of the above assumptions, it was found that the differences in β-glucuronidase activity noted in the three physiological states studied actually represent changes in the enzyme concentration of the uterus.

DISCUSSION

The foregoing studies have clearly established that an elevation in the β-glucuronidase activity of a tissue is not a generalized phenomenon associated with growth *per se* as advocated by Kerr and his associates. This conclusion seems warranted in the light of the following considerations. While ovariectomy in the adult female is associated with a reduction of uterine β-glucuronidase activity (Leonard and Knobil, 1950) the atrophy of seminal vesicle and prostate glands consequent to castration in the male

is not accompanied by a decrease in the enzyme concentration. The β-glucuronidase content of prostates and seminal vesicles was markedly increased, however, when castrated males were treated with estradiol benzoate. The rapid and intense progestational proliferation of the rabbit uterus observed on the fifth day of pregnancy was not paralleled by a rise in uterine β-glucuronidase levels when compared with control tissues removed from these same animals in estrus. Similarly, the β-glucuronidase content of rapidly proliferating deciduomata in the rat, did not significantly exceed that found in the uteri of normal control animals. In these last two cases tissue growth was mediated by progesterone.

Although progesterone markedly inhibited the growth effect of estradiol benzoate on the rat uterus, it failed to depress the estrogen-induced rise in the uterine β-glucuronidase concentration. These results fail to support those of Kerr, Campbell and Levvy (1949 and 1950) who observed that progesterone antagonizes the effect of estrone on the glucuronidase response of the uterus in ovariectomized mice. It may also be inferred from the present experiment that the decrease in β-glucuronidase activity observed on the ninth day of pseudopregnancy was probably due to a decrease in circulating estrogens and not to the inhibitory action of progesterone.

The results obtained from the experiments dealing with immature female rats warrant consideration. In these animals, in contrast to adults, ovariectomy was not accompanied by a decrease in β-glucuronidase activity and estrogen administration failed to effect an increase in enzyme concentration. It may be concluded from this that changes in tissue mass consequent to fluctuations in circulating estrogens are not in all cases accompanied by a change in β-glucuronidase activity.

The injection of testosterone propionate into ovariectomized adult female rats resulted in an increase in uterine weight which was attributable in large part to an increased proliferation of the muscular layers accompanied by an extension of the muscle mass into the mesometrium, as well as an extensive progestational development of the endometrium (Greep and Jones, 1950). When uterine weights of comparable magnitude were induced by the administration of estradiol, the enzyme values were about 50 units per mg. dry weight or nearly double that found following testosterone treatment. This experiment provides another instance wherein tissue growth and enzyme activity were not closely correlated.

A possible interpretation of the increased β-glucuronidase concentration noted on testosterone administration may be that the alteration in enzyme level was actually brought about by estrogens arising from the conversion of testosterone. That such a conversion can occur in the living animal has been rather clearly demonstrated (reviewed by Burrows, 1949). The possi-

bility remains that testosterone directly effected the increase in enzyme activity, but this seems unlikely since it was without effect on the β-glucuronidase concentration of the seminal vesicle and prostate glands.

In the pseudopregnant female rat, deciduomata induced in the right uterine horn were higher in β-glucuronidase activity than the unstimulated left horn within the same animal. Since the deciduomatal tissues employed for enzyme assay in these experiments consisted largely of endometrium while the untraumatized horn comprised a larger proportion of muscle and other components (see Table 8) which, on the basis of histochemical observations, are devoid of β-glucuronidase activity, one would expect that the deciduomatal tissue would contain more enzyme than the control horn if the enzyme concentration in the endometria of both cases remained unchanged. This is indeed what was observed and one may conclude that the difference in enzyme activity under consideration need not be explained in terms of cellular proliferation.

In conclusion, it is believed that the evidence presented here gives considerable substance to the view that changes in the β-glucuronidase activity of the tissues studied may be specifically related to the action of estrogenic hormones.

SUMMARY

The present report deals with studies of the β-glucuronidase activity of the uterus, mammary gland, prostate gland and seminal vesicle in relation to the action of estrogen and other steroid hormones. It was found that castration atrophy of the prostates and seminal vesicles of male rats was not accompanied by a decrease in β-glucuronidase concentration although the administration of estradiol benzoate to castrated males effected a marked rise in the enzyme activity of these organs.

The proliferation of deciduomata in the rat and endometria of rabbits under the influence of progesterone was not paralleled by an increase in β-glucuronidase activity. Ovariectomy in the rabbit and pseudopregnancy in the rat, however, resulted in a decrease in uterine enzyme concentration. Progesterone, when administered to ovariectomized female rats, while effective in antagonizing the growth effects of estradiol benzoate on the rat uterus, failed to inhibit the estrogen-induced increase in uterine enzyme activity.

Testosterone propionate increased the uterine β-glucuronidase concentration of ovariectomized rats to a value which was half that found in uteri stimulated to the same weight with estrogen.

Ovariectomy of immature rats resulted in a decrease in uterine weight which was not accompanied by a decrease in enzyme activity. Conversely, the administration of estrogen to these animals while effecting a 156% in-

crease in uterine weight, was without effect on the uterine enzyme concentration.

The proliferative changes observed in mammary glands of estrogen treated female rats were accompanied by a rise in β-glucuronidase activity, but this hormone was without effect in hypophysectomized females in terms of mammary growth and mammary enzyme concentration. The uteri of the latter animals, however, did respond to hormone treatment by an increase in enzyme activity.

It is concluded that changes in β-glucuronidase activity are not a generalized phenomenon associated with growth *per se* and that the evidence presented lends support to the view that these changes may be specifically related to the action of estrogenic hormones.

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THE EFFECT OF a-ESTRADIOL, SOME OF ITS ESTERS AND ESTRONE ON THE RESPIRATION OF TISSUE HOMOGENATES[1]

MARION A. GUIDRY,[2] ALBERT SEGALOFF AND AARON M. ALTSCHUL

From the Richardson Chemical Laboratory and the Department of Medicine, Tulane University and the Endocrine Research Laboratory, Alton Ochsner Medical Foundation, New Orleans, La.

INTRODUCTION

MEYER *et al.* (1948) showed that certain natural and synthetic estrogens in concentrations of 0.2 to 3×10^{-4} M inhibit tissue respiration by attacking the iron compounds in the cytochrome system. The portion of the estrogenic molecule responsible for this inhibition was traced to the free phenolic groups of these compounds. Case and Dickens (1948) described some synthetic estrogens which inhibit the succinoxidase by acting on the cytochrome portion, some which inhibit by acting on the succinic dehydrogenase system, and still others which inhibit the over-all oxidation of succinate but which act neither on the cytochrome oxidase nor the succinic dehydrogenase.

Data are herein reported which suggest a-estradiol, some of its esters, and estrone in a concentration of 5×10^{-5} M inhibit oxidation of substrates whose oxidative systems are coenzyme linked but do not inhibit the succinoxidase system.

MATERIALS AND METHODS

a-Estradiol (m. p. 176.5–177.5° C.), the 17-propionate (m. p. 200–202° C.), the 3-trimethylacetate (m. p. 185–186° C.), and estrone (m. p. 260–261° C.) were dissolved in alcohol in a concentration of 75×10^{-5} M. Two-tenths of a milliliter of the alcoholic solution was used per flask unless otherwise specified. a-Estradiol-17-hemisuccinate (m. p. 163–164° C.), and the 3, 17-dissuccinate (m. p. 166–167.5° C.) were dissolved in m/75 Sørensen phosphate buffer pH 7.0 at a concentration of 75×10^{-5} M. Lactate, isocitrate, glutamate and succinate substrates were prepared from their acids by neutralization. A 3% solution of glycerophosphate was prepared from a mixture of a and B forms containing 51% and 49% B. Neutralization was not necessary.

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[2] Predoctorate Research Fellow of the National Institutes of Health. Present address, Southwestern Louisiana Institute, Lafayette, La.

Male rats of the Fisher strain ranging in age from 2–4 months and weighing 100 to 200 gm. were decapitated and 1.5 gm. of liver was placed in 30 ml. of m/75 phosphate buffer to which had been added 0.45 gm. of nicotinamide (0.123 M). Homogenization was performed in a cold, all-glass, hand homogenizer.

Oxygen consumption was measured for one hour in Warburg flasks at 37.5° C. at a shaking speed of 80 strokes per minute. The center well of these flasks contained 0.2 ml. of saturated sodium hydroxide and a filter paper strip measuring 22 mm. by 25 mm. rolled in such a fashion that 1 mm. of the paper extended over the top of the center well. Two milliliters of the homogenate and 0.2 ml. of 1.5% substrate were added to the main chamber. The 3% solution of the mixed glycerophosphates was employed as 1.5% a-glycerophosphate. The medium containing the steroid was added to the side arm and water was added to the main chamber to bring the total liquid contents of the flasks to 3.0 ml. After a warm-up period of 5 minutes, the estrogens were tipped in and the manometers closed. This point was considered zero time. Except for figure 3A, the estrogen content in the final incubation mixture was 5×10^{-5} M. The data given are the averages of several experiments and are presented as oxygen consumption for the first hour.

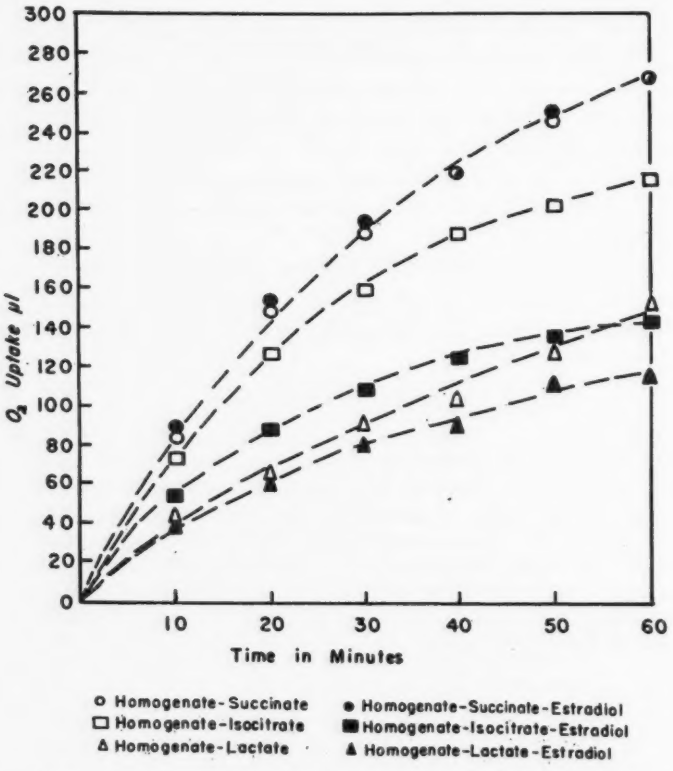

FIG. 1. The O_2 uptake of rat liver mince with added substrate in the presence of a-estradiol.

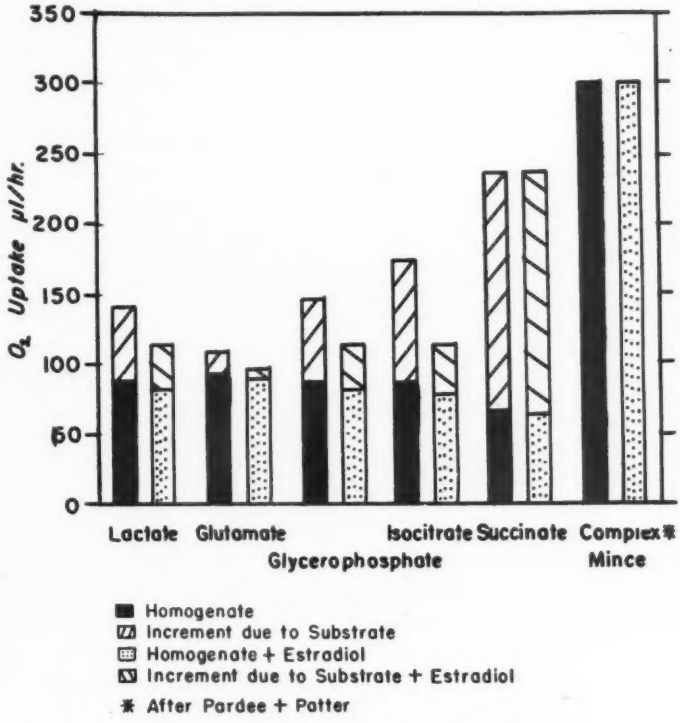

FIG. 2. The O_2 uptake of rat liver mince with added substrate in the presence of a-estradiol.

When the homogenates were studied by the method of Pardee and Potter (1949), the constituents were mixed, placed in the refrigerator, and given ample time for cooling before the animals were killed. Homogenization was then carried out after the liver had been added directly to the mixture.

RESULTS

The rate of oxygen consumption of the rat liver homogenate in phosphate buffer, which was low initially (14 µl at 10 min.), decreased rapidly (16 µl at 20 min.). When the liver was homogenized in buffer containing nicotinamide, the initial rate of oxygen consumption was higher as was the total oxygen consumption during the first hour. The addition of alcohol to the homogenate made in nicotinamide had no significant effect on the oxygen consumption of the medium.

In accordance with the work of Pardee and Potter (1949), the oxygen consumption of the homogenate with added substrates was greatly in-

creased both initially and throughout the first hour. Addition of succinate increased the oxygen consumption of the homogenate, although to a lesser degree than the mixture of substrates according to Pardee and Potter.

Oxygen consumption of the homogenate containing added substrates was significantly decreased by the addition of a-estradiol except in those homogenates containing added succinate or the system adopted from Pardee and Potter (1949). Representative curves are shown in figure 1 and the data are summarized in figure 2. Inhibition of the rate of oxygen consumption was most pronounced in the case of isocitrate. In most cases the oxygen consumption of homogenate with alcohol was not inhibited by a-estradiol.

It is known that a-estradiol is only slightly soluble in water. A suspension of a-estradiol was made, however, in phosphate buffer following the method of Lehninger and Scott (1947), and this suspension was added to the homogenate. With added lactate oxygen consumption was not inhibited (Fig. 3A). Ethanol was added in various concentrations to this suspension to increase the amount of estrogen in solution. Addition of alcohol such that the final medium contained alcohol in 3.5% concentration, and doubling the amount of a-estradiol added, also failed to inhibit oxygen consumption (Fig. 3B). When the alcoholic concentration was increased to 7 and 11%, there was pronounced inhibition when the suspension contain-

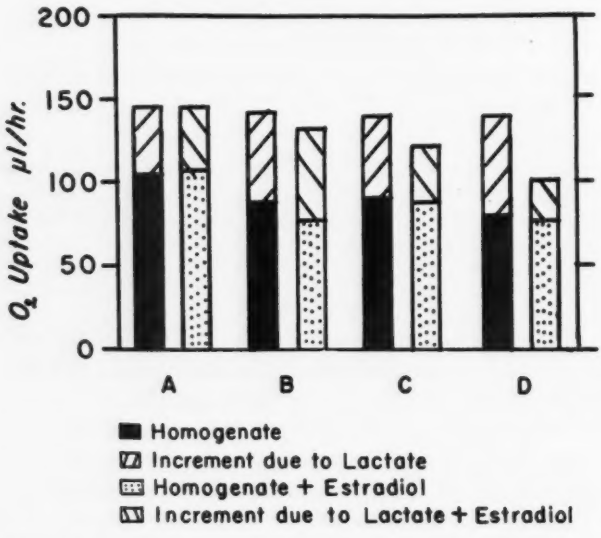

FIG. 3. The effect of soluble a-estradiol as determined by alcoholic concentration.

Experiment	Alcoholic Concentration	a-estradiol added per flask	% inhibition of preparation due to added lactate
A	0%	20 μg.	0
B	3.5%	45 μg.	0
C	7.0%	45 μg.	35
D	11.0%	45 μg.	63

ing a-estradiol was added (Fig. 3, C and D). The amount of inhibition increased with increase in alcoholic concentration. Each experiment was the average of three separate determinations and the blanks contained the same concentration of alcohol as did the solutions containing estrogen.

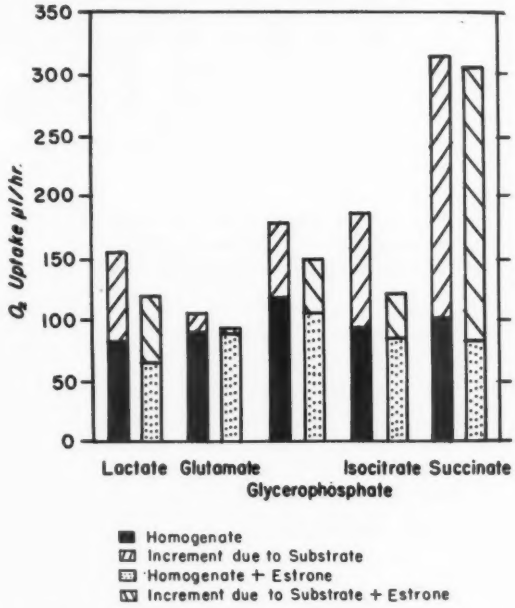

FIG. 4. The O_2 uptake of rat liver mince with added substrate in the presence of estrone.

Estrone showed essentially the same effect as estradiol, inhibiting oxygen consumption of homogenate containing added lactate, glutamate, glycerophosphate and isocitrate but not that of homogenate with succinate (Fig. 4). a-Estradiol-17-propionate and a-estradiol-3-trimethylacetate (Fig. 5) inhibited the oxygen consumption of lactate and isocitrate. The two esters of a-estradiol, the 17-hemisuccinate and the 3, 17-disuccinate were sufficiently soluble in water to enable a study of their effect in non-

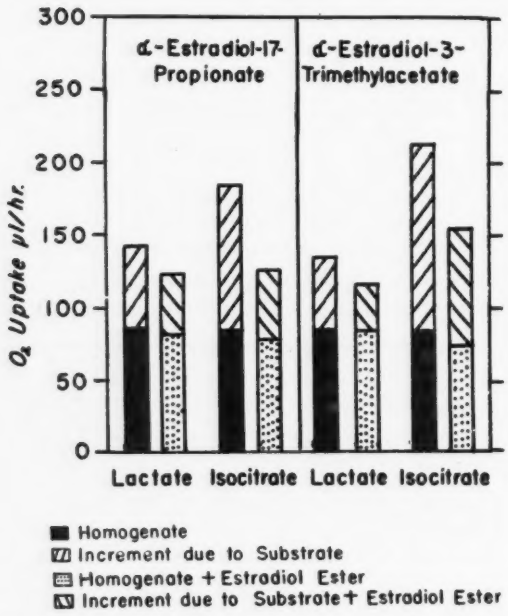

FIG. 5. The O_2 uptake of rat liver mince with added substrate in the presence of estradiol esters.

alcoholic media (Fig. 6). These esters in non-alcoholic media were also found to inhibit oxygen consumption of the homogenate with lactate and isocitrate.

DISCUSSION

The observed oxygen consumption of rat liver homogenate is a composite of oxidation of different substrates. Therefore, the reflected oxygen consumption is a composite of oxygen utilization through several systems of the tissue.

When an excess of one substrate is present, the proportion of its contribution to over-all oxygen consumption is increased. The contribution of the enzyme system involved in the oxidation of the substrate is also, thereby, increased. Thus, when lactate is added in excess in an animal tissue homogenate, the proportion of oxygen consumption utilized through the coenzyme I system is increased. Not all the increased oxygen consumption need be utilized through this system, however, since the products formed from the oxidation of lactate may in turn be oxidized through other systems.

All the above substrates are normally oxidized through the cytochrome

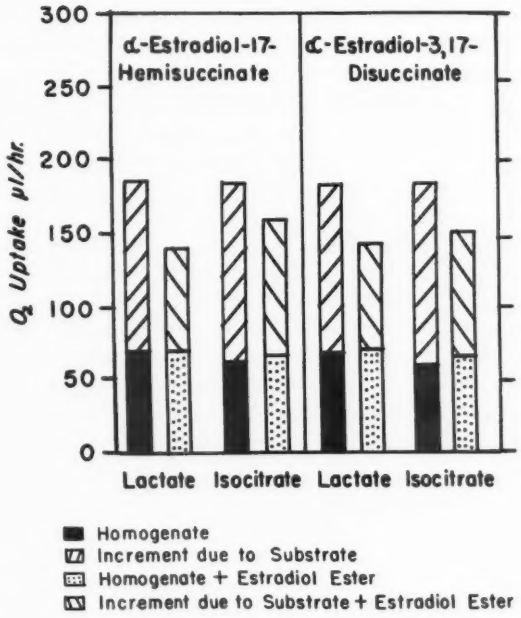

FIG. 6. The O_2 uptake of rat liver mince with added substrate in the presence of estradiol esters.

system. The fact that the extent of inhibition of oxygen consumption by a-estradiol is dependent upon the type of substrate used would suggest that cytochrome c and cytochrome oxidase are not affected by the steroids.

It has been demonstrated that the alcohol used in the above systems enhance the inhibition of respiration by a-estradiol. This action by the alcohol is attributed to its solubilizing effect on the slightly water-soluble steroid. The results demonstrate that a system containing 11% alcohol results in greater inhibition of the oxygen uptake by the steroid than the 7% alcohol system used in general in this work. The latter system was used in preference to the 11% alcoholic system to minimize interference of the oxygen uptake by high concentrations of this agent.

It is significant that the oxygen uptake of the system formulated by Pardee and Potter (1949) to promote and stabilize oxidative phosphorylation by the homogenate is not inhibited by the estrogens. From the data presented in this paper it would seem that the system or systems stabilized by their method did not include coenzyme I and II linked oxidations.

It is suggested that the site of inhibition by a-estradiol and its derivatives is in the systems involving the coenzymes I and II. It has been shown

by Pearlman and Dimeio (1949) and more recently by Ledogar and Jones (1950) that estrone can be isolated from an incubation mixture of a-estradiol and preparations from rat liver. In view of this, the possibility of estrone being the true inhibitor in the present study cannot be overlooked. Also, since there is a possibility that hydrolysis of the esters of a-estradiol occurred, the inhibiting effect of these esters could be due to the free parent estrogen.

SUMMARY

The oxygen consumption of rat liver homogenate with added substrates was studied. Added substrates considerably raised the oxygen consumption of the homogenate. Addition of a-estradiol, some of its esters or estrone decreased the oxygen consumption of homogenates containing those substrates which are oxidized by the intermediation of coenzymes I and II but not that of succinoxidase. Since the estrogens do not inhibit all cytochrome linked oxidations, it has been concluded that this phenomenon is specific for coenzyme I and II linked oxidations.

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CHANGES IN THE ADENOSINE TRIPHOSPHATE AND CREATINE PHOSPHATE CONTENT OF THE RABBIT UTERUS THROUGHOUT SEXUAL MATURATION AND AFTER OVULATION

JOHN H. MENKES[1] AND ARPAD CSAPO[2]

Department of Embryology, Carnegie Institution of Washington, Baltimore

IF ONE accepts the current view that the actomyosin-adenosine triphosphate system is responsible in a final sense for muscular contractility (Mommaerts, 1950; Sandow, 1949; Straub, 1950; Szent-Györgyi, 1951) it seems reasonable to look for possible changes in this system when variation occurs in the contractility pattern of the living muscle itself. Changes occurring at the molecular level might help to understand muscle function as well as the nature of its variation.

For an investigation of this kind the uterus during the course of sexual maturation, menstrual or estrous cycle, and pregnancy offers valuable material. Changes in myometrial contractility in these conditions and the part played by the sexual hormones in producing them have been thoroughly studied with a great variety of experimental animals and techniques *in vivo* as well as *in vitro*. It is impossible even to outline in this paper the published results on this subject and the authors refer the reader for literature to the recent detailed review of Reynolds (1949).

The first step in studying the actomyosin (AM) system quantitatively under different reproductive and hormonal conditions was made by one of us who observed that the AM concentration of human, rabbit and rat uteri increases with advancing pregnancy (Csapo, 1948, 1950a). Similar but more extreme changes in the concentration of the contractile proteins have been observed during embryonic development in chick skeletal muscle (Csapo and Herrmann, 1951). Evidence was also given that following ovariectomy the AM concentration of rabbit uteri and the adenosine triphosphatase (ATP-ase) activity decrease. The administration of estrogen on the other hand initiates a recovery towards the situation existing in estrus (i.e. before ovariectomy) (Csapo, 1950b). It has been concluded from these observations that a quantitative relationship exists (under the conditions studied) between estrogen and AM content. This might be con-

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[1] Student at The Johns Hopkins Medical School.

[2] Lecturer in the Department of Obstetrics, The Johns Hopkins Medical School.

sidered one factor responsible for changes in myometrial contractility during the course of pregnancy, before and after ovariectomy, and after ovariectomy followed by estrogen administration.

The present study, which deals with the phosphorus compounds of the uterus, is a further step in this direction, since not AM but AM-ATP is considered to be the final contractile system. The role of ATP in muscle contraction has been studied extensively on living muscle as well as on models (such as AM-threads, glycerol-treated and washed muscle fibers) with a great variety of techniques. The discussion of these results is beyond the scope of this paper. (For references see Buchthal and Kahlson, 1946; Deuticke, 1932; Engelhardt and Ljubimova, 1942; Szent-Györgyi, 1951).

The importance of ATP in the energetics of muscular contractility was first indicated by Lundsgaard's experiments. He observed (1930) that monoiodoacetic acid (IAA)-poisoned skeletal muscle in nitrogen performs only 100 contractions, whereas the unpoisoned muscle exhibits several hundred. He also demonstrated that the energy liberated by IAA-poisoned muscle in anoxia is proportional to the breakdown of creatine phosphate (PC) and under these conditions ATP disappears on stimulation (1934). In view of these facts and of Lohmann's finding on the P transfer between PC and ATP (1934), and with the help of his conclusion that the breakdown of ATP to adenosine diphosphate (ADP) should precede that of PC, it can be assumed that ATP represents the immediate source of energy for muscular contraction. Since an equilibrium exists between ATP and PC, P transfer proceeds with considerable speed in either direction as determined by the muscle activity itself. It is possible therefore to say that ATP+PC together represent the immediate source of energy for contractility.

ATP has two "high-energy phosphate bonds" (~ph) (Lipmann, 1941) while PC has one such bond, each representing 11,000 calories of free energy per mole if split. This chemical energy can be considered the source of the mechanical energy for muscular work. Although the manner in which this transformation of energy occurs is still unknown, it seems reasonable to study the concentration of high-energy phosphate compounds in relation to uterine contractility.

Preliminary work of this kind has already been reported by Csapo and Gergely (1950). It was observed that the rabbit uterus in estrus contained 1/7 of the high-energy phosphates present in skeletal muscle and also the number of contractions obtained from these uteri in IAA poisoning combined with anoxia was only 1/7 of the number obtained by Lundsgaard in experiments with skeletal muscle. Thus it was concluded that the energy requirement for a single contraction cycle would be of the same order of

magnitude for both kinds of muscle, and in terms of high-energy phosphate bonds it would correspond to about 0.25 μM per contraction per gram. This figure is identical with that calculated by Hill (1949, 1950) on the basis of direct heat measurements of a single twitch of skeletal muscle.

Both ATP and PC have previously been determined in the uteri of different species but without attention to reproductive condition (Eggleton and Eggleton, 1930; Lohmann, 1928; Momigliano, 1932; Sbutega and De Veszelka, 1936; Siracusa, 1929). An extensive study of the high-energy phosphate content of the uterine muscle of rats and rabbits after castration and castration followed by estrogen administration has been reported by Walaas and Walaas (1950). Extremely low values were observed for ATP+PC (0.5 to 0.6 μM per gram of muscle) in both species. These values are about 5 times less than those reported by Csapo and Gergely (1950) for the rabbit uterus in estrus. No direct comparison is possible however between the two figures because one was obtained by observing the isolated muscle layer (of castrated and estrogen-treated animals) and the other from a whole segment of rabbit uterus (in natural estrus). A few comments however may be constructive and will be given below.

METHODS

Experimental animals. Female rabbits from the same stock were used. They were divided into six groups, depending on their age and difference in their reproductive conditions.

1. The term "immature" defines 75 day old animals weighing 1900 ± 100 grams. Sections of the uterine walls and the ovaries show a histological picture typical of this condition. The ovaries contain follicles not larger than 0.6 mm. in diameter. The uterus is 2.5 to 3.5 mm. in diameter, with endometrium containing shallow crypts, but no glands in the deeper part of the stroma. The myometrium is about 0.25 to 0.5 mm. thick, measured opposite the mesometrial attachment.
2. The term "adolescent" is used to describe virgin female animals four months old weighing 2500 ± 150 grams which are not quite mature, but their uteri are definitely more developed than those of the immature group. The histological picture also shows a kind of intermediate stage between immaturity and full maturity (estrus). The uterus is 3.0 to 5.0 mm. in diameter. In addition to shallow crypts near the surface, the endometrium now contains a few glands in the deeper part of the stroma. The surface epithelium is still cubiodal, the myometrium is 0.5 to 1.0 mm. thick.
3. The term "mature, in estrus" means ten month old virgin females weighing 3500 ± 300 grams which were mated 10 minutes previous to the experiment. Successful mating indicates that the animals were in estrus, in agreement with the histological picture of the uterine wall and the ovaries. The ovary contains follicles up to 1 mm. in diameter. The uterus is 1 to 7 mm. in diameter. The endometrial crypts are shallow to deep. The glands in the stroma are numerous. The surface epithelium is low columnar. The myometrium is more than 1 mm. thick.
4. The term "anestrous" is used to describe mature virgin female animals 10 months old, weighing 3500 ± 300 grams, 10 minutes after unsuccessful mating. Theoreti-

cally unsuccessful mating might be due to two causes. One possibility is that prior to mating spontaneous ovulation occurred. (This is considered very rare in rabbits.) Another might be that the natural hormonal stimulus necessary to bring about estrus was inadequate. In our experimental animals the subsequent histological control points to the second possibility. The ovaries contain follicles up to 0.6 mm. The uterus is about 5.0 mm. in diameter. The endometrium contains shallow crypts and few glands in the stroma. The myometrium is 0.6 to 0.75 mm. thick. These uteri therefore are more similar to those grouped as "adolescent," than to those of "estrus" although as far as age and weight are concerned they are similar to the estrous animals.

5. The term "pseudopregnant" refers to ten month old virgin females weighing 3300 ± 300 grams which were mated 3 to 7 days before the experiment to a vasectomized male, thus inducing corpus-luteum formation without impregnation. The histological picture clearly shows the existence of pseudopregnancy, in which condition the uterus is known to be under the dominance of the corpus-luteum hormone, progesterone. This condition is comparable to the progestational period of other animals which ovulate spontaneously. The ovary contains corpora lutea. The endometrium is in the progestational state.
6. The term "pregnant" is used in describing primiparous animals 4000 ± 500 grams which were mated 10 to 21 days prior to the experiment. "At term" rabbits are 32 day pregnant animals a few hours before labor, as indicated by a small amount of bleeding from the vagina.

PROCEDURE

The animals were anesthetized intravenously with Nembutal, 40 mg. per kg. body weight dissolved in Locke solution (20 mg. per ml.). Ten minutes after the injection of the anesthetic, and thus in deep anesthesia, a median laparotomy was performed. First the left ovarian end of the bicornuate uterus was lifted out (by grasping the mesometrium with the forceps) and a segment about 2 cms. long was very quickly cut away from the mesometrium and from the rest of the horn and immersed in a mixture of dry ice and ether. The same was then done with the right horn. The two segments thus obtained were separately but synchronously analyzed later as controls for each other.

The uterine segments were weighted in the deeply frozen state on a torsion balance. The amount of uterus used throughout the whole experiment was about 500 mg. While still in the deeply frozen state, the segments were placed in a glass homogenizer (Potter-Elvehjem type) containing 5 ml. of 5% ice cold trichloroacetic acid. The samples were then homogenized for 1 minute at 0° C. (in an ice-water mixture) at 800 RPM. This homogenization technique was successfully used previously for other purposes (Csapo and Herrmann, 1951). The samples thus obtained were filtered in a refrigerator at −20° C. into an Erlenmeyer flask of 25 ml. capacity which was previously kept for about an hour at this temperature. The drops of trichloroacetic extract leaving the filter paper became frozen immediately

upon reaching the bottom of the flask. After filtration was completed, the samples were kept at room temperature and by shaking the container by hand, they were slowly melted until 0° C. had been reached. Samples for chemical determinations of high-evergy phosphate compounds were taken from this filtrate. Thus the whole procedure went on without delay and the temperature of the samples was always kept at or below 0° C.

Chemical Determination of the Phosphorus Compounds. Inorganic P, inorganic P+PC, and P hydrolyzed in 1 N HCl for 7 minutes were determined. For inorganic P the method of Lowry and Lopez was used (1946). The P in other fractions was determined by the method of Fiske and Subbarow (1925). The difference between inorganic P+PC and inorganic P was expressed as PC, and the difference between the 7 minute labile P and inorganic P+PC as ATP (1 mole ATP per 2 moles labile P). Since ATP and ADP cannot be distinguished by this method it is possible that our ATP values actually represent mixtures (with different ratios) of ATP and ADP. Results were read on a Coleman spectrophotometer at a wave length of 560γ.

RESULTS

As stated above segments from the whole uterine wall were used in these experiments. The uterine wall is composed of 3 layers: peritoneum, myometrium and endometrium. The peritoneum is so thin that it probably does not interfere with the quantitative picture. Since only the myometrium plays an active role in contractility, our interest in this study was concerned mainly with this layer. We isolated the myometrium from the endometrium in a series of experiments in order to learn how this treatment alters the quantitative picture of the highly labile ~ph compounds which they contain.

For this purpose a sample (A) was taken from the living animal as described under *Methods*. Another segment from the same horn was placed with its myometrial side on the glass plate lying on the surface of a solid CO_2-ether mixture and was thus cooled below 0° C. The uterine segment was opened at the mesometrial attachment. The myometrial side (B), being in contact with the cooled plate, immediately froze, but a 1 to 2 minute period permitted the isolation of the endometrium (C) before it became completely hard (due to complete freezing). After isolation of the two layers they were immersed in the solid CO_2-ether mixture. As a control sample, another segment (D) was also placed on the cooled glass plate and kept there in anoxia during the course of separation of the two layers described previously. For time control, finally, a sample (E) was kept at 0° C. for 10 minutes in oxygenated Locke solution, then immersed in the freezing mixture. In the following steps the standard technique (see

Methods) was used for all samples. The results obtained are summarized in Table 1.

One can see from these results that the highest ~ph value is obtained from the living animal. A slight decrease occurs even after 10 minutes at 0° C. in oxygenated Locke solution. Both the myometrium and endometrium show extremely low values if separated with no PC in the endometrium. The control sample which was kept under similar conditions but was not subjected to separation, shows considerably higher values than the separated layers, but still lower than those obtained from the living animal or after ten minutes at 0° C. in oxygenated Locke solution.

TABLE 1. CHANGES IN CONCENTRATION OF THE PHOSPHORUS COMPOUNDS OF RABBIT UTERUS DURING THE PROCEDURE OF SEPARATION OF ITS LAYERS. ALL VALUES IN MICROMOLES PER GRAM TISSUE. (SEE TEXT FOR DESCRIPTION.)

Reproductive condition	P compounds μM per g. tissue	A	B	C	D	E
18 days pregnant	Inorganic P	3.26	4.71	3.99	4.38	2.84
	PC	2.28	0.51	0.00	—	1.61
	ATP	3.56	1.60	1.50	1.58	3.13
Mature nonpregnant	Inorganic P	3.38	4.71	3.26	4.02	3.07
	PC	0.94	0.84	0.10	0.64	0.84
	ATP	3.29	1.21	0.87	2.62	2.86
Mature nonpregnant	Inorganic P	3.90	4.28	3.03	3.93	2.84
	PC	1.26	0.74	—	1.06	2.58
	ATP	1.81	0.84	0.42	1.94	1.27

As an additional experiment we took a sample directly from the living animal (No. 1) and kept 6 pieces from the same uterus under different conditions. One was left at 0° C. (No. 2) and another at 37° C. (No. 3) for 15

TABLE 2. CHANGES IN CONCENTRATION OF THE ATP+PC CONTENT OF THE UTERUS DUE TO ANOXIA AND SUBSEQUENT OXYGENATION. ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No.	Condition previous to determination	ATP+PC μM per 1 g. tissue
1	Living animal	3.33
2	15 min. anoxia at 0° C. in Locke solution	1.31
3	15 min. anoxia at 37° C. in Locke solution	0.68
4	15 min. anoxia at 0° C. in Locke solution and 15 min. subsequent oxygenation at 0° C.	1.63
5	15 min. anoxia at 37° C. in Locke solution and 15 min. subsequent oxygenation at 0° C.	1.18
6	30 min. at 0° C. in oxygenated Locke solution	3.32

minutes in Locke solution without oxygenation. After 15 minutes one half of each of these samples was placed in the freezing mixture; the other half was immersed in an oxygenated Locke solution at 0° C. for another 15 minutes (Nos. 4 and 5). A sample (No. 6) was also taken from the living animal and kept at 0° C. for 30 minutes in oxygenated Locke solution. The results are summarized in Table 2.

One can see from Table 2 that there is no significant difference between Samples 1 and 6, but anoxia results in a considerable loss to the ~ph content even at 0° C. This decrease can be only slightly reversed by subsequent oxygenation. These experiments suggest that during preparation the ~ph compounds of the uterus decrease even at 0° C. if the muscle is kept in anoxia. Further loss in these compounds occurs if the samples are

TABLE 3. CONCENTRATION OF PHOSPHORUS COMPOUNDS IN THE UTERI OF IMMATURE RABBITS (75 DAYS OLD). ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No.	Inorganic P	PC	ATP	Inorganic P +PC+ATP	PC+ATP
167	2.93	0.61	2.05	5.59	2.66
	3.06	0.16	1.82	5.04	1.98
168	2.96	0.06	2.34	5.36	2.40
	4.44	0	2.45	6.89	2.45
169	2.45	0	1.27	3.72	1.27
	3.38	0	1.97	5.35	1.97
Average	3.20	0.14	1.98	5.33	2.12

handled with scissors or razor blades (mechanical irritation?) during the process of separation of the layers. We therefore used whole uterine segments in the following experiments, taking samples directly from the living animal.

We turn now to our findings on the ~ph content of the rabbit uterus in different reproductive conditions. As shown in Table 3 the undeveloped uteri of immature rabbits (see description under *Methods*) contain the lowest ~ph concentration observed in this series of experiments.

The high inorganic P and low (almost nil) PC values are conspicuous. The control samples are in good agreement.

Table 4 shows that the ~ph concentration of adolescent rabbit uteri is about 50% higher than that of the immature animals.

In comparison with immature animals the inorganic P in this group is lower and PC appears in considerable quantity. No significant change can be seen in ATP concentration. Controls are in agreement.

The uteri of estrous rabbits (Table 5) contain almost twice as much ~ph as is present in the uteri of immature rabbits.

TABLE 4. CONCENTRATION OF PHOSPHORUS COMPOUNDS IN THE UTERI OF ADOLESCENT RABBITS (4 MONTHS OLD). ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No.	Inorganic P	PC	ATP	Inorganic P +PC+ATP	PC+ATP
151	2.19	1.94	1.84	5.95	3.76
	2.19	1.81	1.82	5.82	3.63
152	2.61	0.87	1.78	5.26	2.65
	2.38	0.64	1.98	5.00	2.62
153	2.26	1.10	2.16	5.52	3.26
	2.00	1.00	2.35	5.35	3.35
154	2.48	0.48	1.66	4.62	2.14
	2.68	0.39	1.79	4.86	2.18
155	3.19	1.49	1.24	5.92	2.73
Average	2.44	1.08	1.85	5.37	2.92

TABLE 5. CONCENTRATION OF PHOSPHORUS COMPOUNDS IN THE UTERI OF MATURE RABBITS IN ESTRUS (10 MONTHS OLD). ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No.	Inorganic P	PC	ATP	Inorganic P +PC+ATP	PC+ATP
156	3.38	1.29	2.33	7.00	3.62
	2.87	0.78	2.48	6.13	3.26
157	2.58	1.19	2.56	6.33	3.75
	2.93	1.61	2.21	6.75	3.82
158	3.61	1.03	2.00	6.64	3.03
	2.56	1.36	1.87	5.81	3.23
159	3.09	1.29	2.55	6.93	3.84
	2.12	1.52	3.21	6.85	4.73
Average	2.90	1.26	2.40	6.56	3.66

TABLE 6. CONCENTRATION OF PHOSPHORUS COMPOUNDS IN THE UTERI OF MATURE RABBITS IN ANESTRUS (10 MONTHS OLD). ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No.	Inorganic P	PC	ATP	Inorganic P +PC+ATP	PC+ATP
165	2.74	1.13	2.42	6.29	3.55
	2.48	1.26	2.26	6.00	3.52
166	2.22	0.61	1.63	4.46	2.24
	1.81	0.71	1.79	4.31	2.50
Average	2.31	0.93	2.02	5.26	2.95

Both ATP and PC values are higher than in the uteri of immature or adolescent rabbits. Controls are in agreement.

The uteri of rabbits in anestrus show (Table 6) ~ph values similar to those of the adolescent.

Both inorganic P and ~ph values in this group are similar to those of adolescent animals, yet these anestrous animals were similar to those in estrus (age, weight, stock) except that mating previous to the experiment was unsuccessful. It seems interesting to note that these uteri were also histologically similar to those of adolescence and not those in estrus.

The uteri of pseudopregnant rabbits contain even higher values of ~ph compounds than those in estrus (Table 7).

TABLE 7. CONCENTRATION OF PHOSPHORUS COMPOUNDS IN THE UTERI OF MATURE RABBITS IN PSEUDOPREGNANCY (10 MONTHS OLD). ALL VALUES IN MICROMOLES PER GRAM UTERUS. (SEE TEXT FOR DESCRIPTION.)

No. and days of pseudopregnancy	Inorganic P	PC	ATP	Inorganic P +PC+ATP	PC+ATP
171–3 days	3.38	0.64	3.61	7.63	4.25
	2.77	0.64	3.61	7.02	4.25
161–5 days	2.19	1.74	2.58	6.51	4.32
	2.42	1.48	3.09	6.99	4.57
162–5 days	1.29	1.35	2.80	5.44	4.15
160–6 days	2.38	2.67	2.32	7.37	4.99
	1.84	1.74	2.85	6.43	4.59
164–7 days	1.77	1.20	2.88	5.85	4.08
	2.09	1.03	2.40	5.52	3.43
Average	2.24	1.39	2.90	6.52	4.29

The values of both ATP and PC are higher in the uteri of this group than in those of estrous animals. The inorganic P is lower. Controls are in agreement.

DISCUSSION

We used whole rabbit uterine segments in our series of experiments and compared the ~ph content of these uteri in different reproductive conditions. We were primarily interested in the ~ph content of the myometrium, this layer being responsible for uterine contractility. We found however that (with the techniques now available) the separation of the myometrium from the endometrium results in about 50% loss in the ~ph compounds as compared with the whole uterine wall. While this loss is due to the preparation, in rabbits (and probably in other animals, which have similar uteri, such as the rat, guinea pig, and sow) no separation of the myometrium from the endometrium is possible without considerable loss

to their ~ph content. This is rather unfortunate as these animals are those most commonly used in reproductive physiology. If we suppose that the decrease in the two layers is identical during the period of separation, then our results indicate that the ATP content of the uterus *in vivo* is lower in the endometrium than in the myometrium. PC cannot be found in the separated endometrium at all, an observation which may suggest that this layer does not contain PC under *in vivo* circumstances. This conclusion is rather indirect however. It seems likely that our ~ph values for the whole uterine segments are not higher but slightly lower than the actual ATP and PC content of the myometrium should be, since the endometrium seems to contain less ATP (and no CP) than the myometrium does if separated. It is possible that the endometrium has a variation (during sexual maturation, cycle and pregnancy) in its ATP content, but this variation and the exact ratio in the ~ph compounds between endometrium and myometrium cannot be studied at present in rabbits (with the techniques described previously). In humans and monkeys this study might be possible however since both layers are thicker and samples can probably be collected from the living individual.

The presence of ATP in human myometrium and endometrium has actually been observed, and a quantitative study is under way with respect to the possible changes of the ~ph concentration in the two uterine layers, throughout menstrual cycle and pregnancy. (Personal communication, Dr. V. A. Fullilove.)

On the basis of our study we suggest that the low ~ph values obtained by Walaas and Walaas (1950) on the isolated myometrium of the rat are partly due to a loss of ~ph compounds during preparation. On uterine segments of rats (5 animals near sexual maturity) we obtained an average value for ~ph of 1.5 μM per gram of tissue. In view of a 50% loss in the high-energy phosphate compounds during the separation process (as indicated by Table 1), this value decreases to 0.75 μM per gram, a figure which is similar to their finding (0.5 to 0.6 μM per gram myometrium). This is an indirect calculation however, since our technique was probably not quite identical with their experimental methods.

We obtained an average value for ~ph in the uteri of estrous rabbits of 3.6 μM per gram. This figure is higher than that obtained previously by Csapo and Gergely (1950) (2.6 μM per gram). The discrepancy might be due to differences in the rabbit colony used, but more likely results from slight differences in preparatory technique. Our ATP values for rabbit skeletal muscle (psoas) is 10 μM per gram; the PC is 19 μM per gram on the average.

During the course of sexual maturation we found 73 per cent increase in the ~ph concentration of the uterus of the rabbit. It seems to be note-

worthy that the uterus of the immature animal does not contain a significant amount of PC. The uterus of the immature rabbit is known to be inactive (no spontaneous contractility) and therefore presumably does not require ~ph compounds for contractility, though it may need those substances for other purposes. As a result of estrogen administration to the living animal, these uteri can be brought into spontaneously active condition. It would be interesting to learn whether such treatment would result in changes in the ~ph content of the uterus in general and in the PC content in particular. Walaas and Walaas (1950) reported that a significant increase in the ~ph content of the myometrium in castrated and subsequently estrogen-treated rats does not occur. Since increase in the ~ph concentration of the uterus of rabbits during sexual maturation is reported in this paper, more detailed study of this question is indicated.

Thus far we are able to say only that the natural stimulus of sexual maturation results in an increase in the ~ph content of the uterus and whether an increased estrogen production is solely responsible for this change cannot be stated as yet.

In estrus the rabbit contains about 8 times less ~ph compounds (3.6 μM per gram tissue) than present in the skeletal muscle (psoas) of the same species (29.0 μM per gram muscle). A similar ratio was observed by Csapo and Gergely (1950). It might be interesting to mention that a similar ratio was found for AM in the two types of muscle (Csapo, 1950a; Szent-Györgyi, 1945, 1951). This similarity in ratios for ATP and AM between skeletal and uterine muscle suggests that from a quantitative point of view a stoichiometric relation which is similar in both kinds of muscles exists in the final contractile system (AM:ATP). On the basis of this fact and other considerations (such as unchanged speed of the *in vitro* uterine contraction when the ATP concentration decreases in the course of fatigue; great differences in the speed of thread contraction of skeletal and uterine AM in the presence of similar ATP concentration) the authors hesitate to accept the view (Walaas and Walaas, 1950) that the low ATP content of the uterus is responsible for the slowness of the uterine contraction cycle. It is more likely that the low activity of those (enzyme?) systems which are involved in the excitatory, contraction and relaxation processes is responsible. These systems are largely unknown at present in the uterus, for in addition to a few studies on the ATP-ase activity (Csapo, 1949, 1950b), only some viscosimetric observations are available (Csapo, 1949) which indicate that the reaction between extracted AM and ATP is slower in the case of uterine than of skeletal muscle. Studies with AM-threads (uterine and skeletal) point to the same conclusion (Csapo, 1948; 1950a).

After ovulation (i.e. in pseudopregnancy) still further increase was observed in the ~ph concentration of the rabbit uterus. Whether this in-

crease actually occurs in the myometrium or is due to progestational proliferation of the endometrium (increase in the glycogen content of the endometrium has been observed in this condition; see for literature Reynolds, 1949) cannot yet be stated. No further comment is possible therefore on this point.

In this section the authors would like to present some problems in regard to ~ph concentration during pregnancy instead of assumptions, because the experimental data are not yet complete. The figures listed here were not presented under *Results* on account of certain technical difficulties involved in the problem. In pregnancy we obtained ~ph values in the uteri of 12 rabbits. The variation at the same day of pregnancy between individuals and samples, greater than that found in the nonpregnant uteri, indicates that the technique is not yet adequate for this purpose. The variation might be due to the presence of attached fetal tissue or to the great sensitivity of the muscle to mechanical stimulation. The highest ~ph concentration in the course of these experiments was obtained from 18-day pregnant uteri (4.8 to 5.8 μM per gram uterus). At 20 to 21 days of pregnancy however the average value is definitely lower (2.8 μM). At term similar values were obtained (3.3 μM). It remains to be seen therefore whether a change in the ~ph concentration of the rabbit uterus occurs around day 20 in pregnancy or this decrease is simply a technical artifact. Such a change would be interesting in view of the description by Reynolds (1949) of general alterations in the pregnant uterus about this time. The authors present these values as indications of the difficulties involved in studying the ~ph concentration of the uterus during pregnancy. Because of its extreme physiological importance this problem calls for further attention and more detailed study.

Finally we may ask what are the physiological implications of the observed ~ph concentration changes of the uterus in myometrial contractility. The answer to this question is limited by the fact that the role of ATP in muscle function in general is not fully clear up to this time. On the basis of Lundsgaard's and Lohmann's evidence we may suggest that increase in the ~ph concentration during sexual maturation in the uterus is one factor in the increase in its working capacity. Since more ATP was found both in skeletal and uterine muscle than is required to supply the energy for one contraction cycle, it is possible that a certain amount of this substance is necessary to make the contractile system "complete and reactive" (Szent-Györgyi, 1949).

Beside the observed increase in the ~ph and AM concentration one would expect other changes to occur in the myometrium during sexual maturation in view of the marked difference in physiological behavior in immature and estrous animals. *In vitro* experiments in progress in our labo-

ratory which will be reported elsewhere show for example that the number of contractions in anoxia is greatly increased during sexual maturation and pregnancy. This indicates a great change in the glycogen content under these conditions (Csapo and Corner, unpublished). These observations are in good agreement with the recent findings that the rat uterus after castration contains 8 μM, after estrogen treatment 20 μM, and in pregnancy 28 μM glycogen (O. Walaas, personal communication). Since $\sim$ph is mostly generated from glycogen in muscle, an increase in glycogen content of the myometrium means a potential increase in $\sim$ph.

To extend and complete this study experiments are under way with Kalckar's enzyme technique, in order to determine the concentration of ATP, ADP, and AMP in the rabbit's uterus in different reproductive conditions.

SUMMARY

Separation of the myometrium from the endometrium of the rabbit uterus was carried out in order to determine the concentration of the phosphorus compounds present in the separate layers. It was observed that this procedure results in about 50 per cent loss in the high-energy phosphate compounds of the whole uterine wall. This technique therefore does not permit quantitative conclusions with respect to the separate layers. It can be said, however, that while the myometrium contains both adenosine triphosphate and creatine phosphate, the endometrium seems to contain only adenosine triphosphate.

Analyzing whole uterine segments taken from living rabbits, we observed an increase in the high-energy phosphate compounds throughout sexual maturation and after ovulation. While in case of nonpregnant uteri the data obtained were in agreement, during pregnancy there was a great variation in the high-energy phosphate values, probably resulting from technical difficulties.

The physiological significance of the observed changes in the high-energy phosphate content of the rabbit uterus is discussed.

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THE INFLUENCE OF CORTISONE ON RENAL FUNCTION AND ELECTROLYTE EXCRETION IN THE ADRENALECTOMIZED DOG[1]

KATHLEEN E. ROBERTS[2] AND ROBERT F. PITTS
with the technical assistance of Muriel V. Seip
and Margaret H. Kennedy
Department of Physiology, Cornell University Medical College, New York, N. Y.

ONE OF the most important renal deficits, which has been accepted as a constant finding in adrenal insufficiency, is the inability of the kidney to limit sodium excretion when normal or subnormal quantities of salt are ingested (Loeb, 1941; Roemmelt *et al.*, 1949). In addition to the renal wastage of sodium, there is frequently a decreased rate of glomerular filtration and a depression of the renal plasma flow which accompanies adrenal insufficiency (Guadino *et al.* 1949, Harrison *et al.*, 1939, White *et al.*, 1947). As a result of such renal functional deficits, there results a decrease in plasma sodium and an elevation of plasma potassium levels as well as a progressive depletion of extracellular fluid volume (Loeb, 1941; Smith, 1951). It is generally accepted that the administration of desoxycorticosterone, either to adrenalectomized animals or to patients with Addison's disease, corrects the renal deficit in sodium excretion as well as the abnormal plasma levels of sodium and potassium (Barnett *et al.*, 1949, Ferrebee *et al.*, 1939, Loeb 1941). It has also been reported that adequate desoxycorticosterone therapy restores toward normal the decreased glomerular filtration rate and renal plasma flow which occurs in adrenal insufficiency (Guadino *et al.*, 1949, Waterhouse *et al.*, 1948, White *et al.*, 1947).

The influence which cortisone exerts on renal function and in correcting the renal deficits seen in adrenal insufficiency has not been so clearly established. Earlier reports have claimed that cortisone administration resulted in an increased renal excretion of sodium (Gaunt *et al.*, 1949; Thorn *et al.*, 1941). From this, it has been postulated that desoxycorticosterone and cortisone are antagonistic as regards the excretion of sodium (Thorn *et al.*, 1941). More recently however, as a result of the widespread use of cortisone in a variety of clinical conditions, it has been observed that so-

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dium retention may occur following the administration of cortisone (Baehr *et al.*, 1950, McEwen *et al.*, 1950, Perera *et al.*, 1949, Russel 1950, Sprague *et al.*, 1950).

To define more clearly the effects of cortisone on renal function and to determine whether there was an antagonism between this hormone and desoxycorticosterone, particularly in their effects on sodium excretion, the series of experiments, described below, were done. In these experiments the effects of these two steroids on renal function and electrolyte excretion in adrenalectomized dogs were compared. The results of these experiments indicate that cortisone and desoxycorticosterone have similar effects on the renal excretion and plasma concentration of sodium and potassium and that these steroids also affect glomerular filtration rate and renal plasma flow in the same direction.

METHODS

All experiments were performed on bilaterally adrenalectomized dogs. During the immediate postoperative period and in the interval between experiments the dogs were maintained on daily injections of desoxycorticosterone[3] (1 mg.) and cortisone (10 mg.). For 3–5 days previous to the experimental period, all supportive measures other than free access to .6 per cent saline, were withdrawn. During a majority of the experiments, the animals were in a state of mild or moderately severe insufficiency.

The experiments were performed in the following manner; the animals were loosely restrained on an animal board; creatinine and para-aminohippurate, in amounts sufficient to maintain optimum plasma levels, were infused at a constant rate. Following an adequate control period, the animals were given desoxycorticosterone, cortisone, or both steroids intravenously and the effect of the steroid on the renal clearances of creatinine and para-aminohippurate, as well as sodium and potassium excretion determined over a two to three hour period. In a few instances more prolonged determinations on renal function and electrolyte excretion were made. Desoxycorticosterone was given as the crystalline preparation dissolved in sesame oil and homogenized with water. Cortisone acetate was given as a microcrystalline suspension in saline. Sodium and potassium values on plasma and urine were determined with an internal standard flame photometer. Creatinine and para-aminohippurate were determined colorimetrically by suitable methods (Bonsnes *et al.* 1945, Smith *et al.* 1945).

RESULTS

Effects of intravenous cortisone on sodium excretion

Table 1 shows the results of intravenous injection of cortisone; the most striking effect is a decrease in sodium excretion which begins within the first few minutes. If the amount of filtered sodium is calculated as a product of the glomerular filtration rate and plasma sodium level, it is possible, by

[3] We are indebted to the Schering Corporation for the generous supply of desoxycorticosterone used in these experiments.

TABLE 1. EFFECTS OF INTRAVENOUS ADMINISTRATION OF CORTISONE AND DESOXYCORTICOSTERONE

Time	Urine flow	Glom. filt.	R.P.F.	Sodium				Potassium		
				Plasma	Urine			Plasma		Urine
min.	cc./min.	cc./min.	cc./min.	mM/L	mM/L	micro eq./min.	% Reabsorbed	mM/L	mM/L	micro eq./min.
Control	.7	63.7	244	135	206	144	98	4.9	109	76.5
				Cortisone, 20 mg. intravenously						
30	.5	63.2	240	135	88.6	44	99.3	4.4	105	52.5
60	.4	60.7	299	135	47.6	19	99.9	3.87	105	42
90	.3	64	253	133	27.3	8.0	99.9	4.01	104	31
120	.2	65.7	212	130	24	5.0	99.9	3.84	147	30
150	.28	62.0	224	130	45	12.6	99.9	3.84	124	34
Control	1.3	70	258	141	261	340	96.5	4.6	63	82
				Desoxycorticosterone, 1 mg. intravenously						
30	1.1	68	270	140	201	221	96.7	4.4	96.4	106
60	.37	63	271	140	85	31.4	99.5	3.5	144	53
90	.46	61	313	140	29	13.3	99.9	3.5	130	60
120	.51	69.5	300	140	41	21	99.9	3.6	147	74.9
150	.6	71.5	292	140	47	28	99.9	3.36	129	77.4

subtracting the excreted amount, to determine the approximate percentage of filtered sodium which is reabsorbed by the kidney tubules. It is then apparent that the decrease in sodium excretion, following the administration of cortisone, is the result of an increased reabsorption of this ion; the percentage of filtered sodium which is reabsorbed by the kidney tubule increasing from 98 to 99.9 per cent. This percentage change in sodium excretion could account for the conservation of some 144 milliequivalents of sodium in twenty-four hours, and is the equivalent of that amount of sodium found in one liter of extracellular fluid.

That the intravenous administration of desoxycorticosterone acetate also resulted in a decrease in sodium excretion is shown in Table 1; the effects of this steroid on sodium did not differ essentially from that seen following the injection of cortisone.

That cortisone and desoxycorticosterone have similar effects on the renal excretion of sodium is illustrated in Fig. 1. Not only do these two steroids have qualitatively similar effects on sodium excretion, but alterations in the dosage of either steroid affected sodium excretion in a quantitatively similar fashion. If the decrease in sodium excretion is calculated as the per cent change from the control level, it is possible to quantitate the dosage of hormone with the change in sodium excretion; the percent decrease over a two hour period, following the injection of steroid, ranging from around 40 percent with the smaller doses to 80 per cent with larger amounts. To further test the hypothesis that cortisone and desoxycorticosterone had a similar effect on sodium excretion, a series of experiments were done to determine if these two steroids had an additive effect in reducing sodium excretion. To do this it was necessary to inject an amount of each steroid which, when given alone, was just sufficient to reduce sodium excretion by a minimal amount; administration of both cortisone

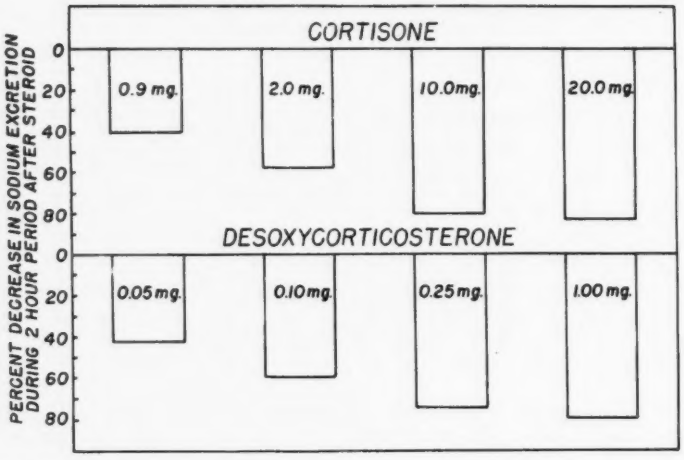

FIG. 1. Comparison of the decrease in sodium excretion obtained with various amounts of cortisone and desoxycorticosterone.

and desoxycorticosterone together, would then have an effect which would be double the magnitude seen when each was given alone. Fig. 2 illustrates this additive effect of cortisone and desoxycorticosterone on the absolute decrease in sodium excretion over a two-hour period; the intravenous injection of both steroids at the same time, resulting in a decrease in sodium excretion which was greater than the decrease caused by each agent alone. This additive effect of cortisone and desoxycorticosterone on sodium ex-

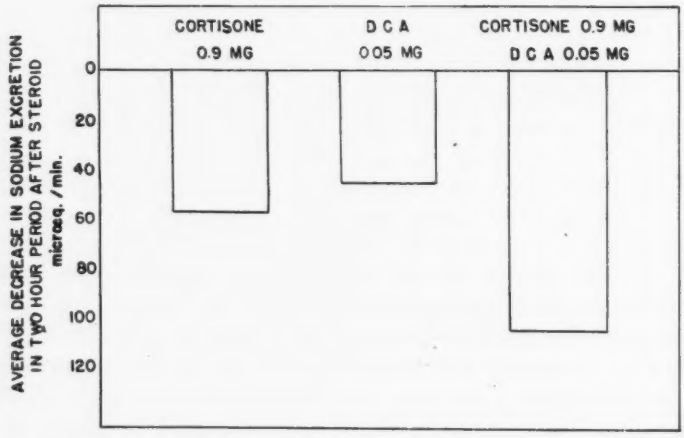

FIG. 2. Additive effect of cortisone and desoxycorticosterone on sodium excretion.

cretion becomes apparent only with the smaller dosage since each steroid has only minimal effects at this dose level. With the larger doses of steroid, where the effect of each on sodium excretion is maximal, no additive effect can be measured. On the other hand, it was evident that cortisone and desoxycorticosterone even in maximum quantities did not have opposite effects on the renal excretion of sodium.

Glomerular filtration rate and renal plasma flow

That the creatinine clearance, which was used as a measure of glomerular filtration rate was not significantly altered during the 2–3 hour period following the administration of cortisone is seen in Tables 1 and 2. Table 1 shows the effects of cortisone and desoxycorticosterone on glomerular filtration rate and renal plasma flow at a time when the dogs had been without therapy for two days but were not yet in a state of insufficiency. Table 2 contains the data on the effects of cortisone and desoxycorticosterone at a time when the animals were in a state of moderately severe insufficiency

TABLE 2. EFFECTS OF CORTISONE ON GLOMERULAR FILTRATION RATE AND RENAL PLASMA FLOW DURING INSUFFICIENCY

Time	Glom. filt rate	R.P.F.
minutes	cc./min.	cc./min.
	I	
Control	29.9	116
	Cortisone intravenously	
120	33.1	159
	II	
Control	37.7	157
	Cortisone and doca	
120	38.4	161

with decreases in the rate of glomerular filtration and renal plasma flow. In neither instance was there any significant effect on the glomerular filtration rate within the two hour period following the injection of cortisone. However, measurement of the glomerular filtration rate over a longer period of time, following the administration of cortisone and during periods of adequate maintenance with this steroid, showed that cortisone was effectual in increasing the depressed filtration rate seen in insufficiency. Table 3 shows the glomerular filtration rate of two dogs measured during periods of insufficiency and when the animals were on hormone therapy. It can be seen that during periods of insufficiency the filtration rate was depressed, and with adequate hormonal replacement the glomerular filtration rate was well maintained. The effects of cortisone on para-aminohippurate clearance as a measure of renal plasma flow are also illustrated in Tables 1, 2 and 3. Although the para-aminohippurate clearance was

TABLE 3. GLOMERULAR FILTRATION RATE AND RENAL PLASMA FLOW DURING INSUFFICIENCY AND WHILE ON ADRENAL CORTICAL THERAPY

Date	Therapy	Glom. filt. rate	R.P.F.
		cc./min.	cc./min.
	Dog. 16		
3/ 9/51	0	33.5	157
3/21/51	0	29.5	116
4/13/51	Cortisone and DCA	60	190
5/10/51	Cortisone	62.5	306
7/13/51	Cortisone	62	260
	Dog D		
7/ 2/51	Pre-adrenalectomy	69	210
	Adrenalectomized		
7/19/51	0	51	196
7/23/51	Cortisone	89	325

slightly elevated as a result of cortisone injection as shown in Tables 1 and 2, the effect was transient and slight during the immediate two-hour period after steroid injection. Measurement of the clearance of para-aminohippurate during periods when the animals were on cortisone therapy showed that cortisone was equally effective in increasing the clearance values of para-aminohippurate as it was in restoring the creatinine clearance values.

The immediate effects of desoxycorticosterone on the glomerular filtration rate and renal plasma flow during the three-hour period following intravenous injection were similar to those seen after cortisone administration.

Potassium

Following the withdrawal of hormone therapy, there was a gradual rise in plasma potassium level. This elevation occurred only when the plasma sodium level was decreased. Figure 3 shows this relationship between the decrease in plasma sodium and the increase in plasma potassium, which was seen following the withdrawal of therapy. Administration of cortisone, desoxycorticosterone or both cortisone and desoxycorticosterone together, resulted in a return to normal of the plasma levels of both sodium and potassium. In this respect both steroids served equally well in maintaining normal plasma concentrations of sodium and potassium.

Renal excretion of potassium, following the withdrawal of steroid, also increased concomitantly with the rise in plasma potassium. This increased renal excretion of potassium was seen, however, only in the first stages of insufficiency; as the animals approached a condition of extremis, the renal

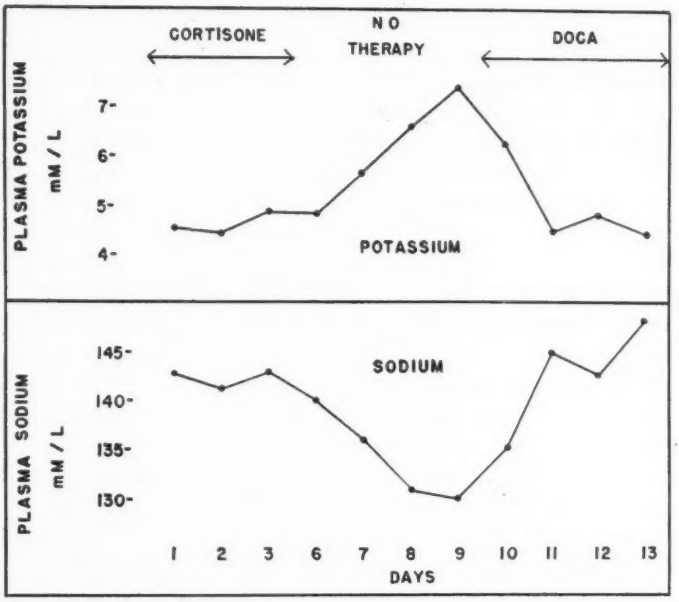

FIG. 3. Relationship between plasma, sodium and potassium during adrenal insufficiency and recovery.

excretion of potassium fell, as did the glomerular filtration rate, renal plasma flow and urine excretion.

The effects of cortisone administration on the renal excretion of potassium were neither so consistent nor so striking as were the effects on sodium excretion. Although a decrease in the elevated plasma potassium level was consistently measured following cortisone administration, this was not always accompanied by an increased urinary excretion of this ion. Table 1

TABLE 4. POTASSIUM EXCRETION FOLLOWING CORTISONE ADMINISTRATION

Time	Potassium			Urine flow
	Plasma	Urine		
minutes	mM/L	mM/L	microeq/min.	cc./min.
		(1)		
Control	6.55	54.0	90.0	1.66
		Cortisone		
120	4.43	107.5	82	.76
		(2)		
Control	6.36	136.5	123	.9
		Cortisone and desoxycorticosterone		
120	4.15	200	120	.6

shows the results on plasma potassium level and renal potassium excretion following the administration of cortisone and desoxycorticosterone. Administration of the steroid at a time when the plasma level is elevated resulted in a drop in plasma concentration which is well defined within a two hour period. Although the concentration of potassium in the urine became elevated following the administration of adrenal steroid, the total excretion could not be correlated with the decrease in plasma level.

DISCUSSION

From the above data, it is evident that cortisone has an important influence in the correction of the renal functional defects as well as deranged electrolyte metabolism which occurs in adrenal insufficiency. Furthermore, the effects of cortisone and desoxycorticosterone administration in the adrenalectomized animal are similar and additive. These findings are in agreement with the numerous observations that cortisone administration results in sodium retention both in the patient with intact adrenals and in Addison's disease (Baehr *et al.*, 1950, McEwen *et al.*, 1950, Perera *et al.*, 1949, Sprague *et al.*, 1950).

Although the dosage of cortisone which was found necessary to produce the same effects as desoxycorticosterone was some fifteen to twenty times greater, this does not invalidate the conclusion that these two steroids have similar effects on the renal functions measured in these experiments. Comparison of the dosage of cortisone used in our experiments with amounts found to be released from adrenals of dogs is quantitatively similar (Vogt, 1943). The amounts of cortisone necessary to produce minimal and maximal effects on sodium excretion are within the dosage range which has been found necessary to protect adrenalectomized animals from a variety of stress and strain conditions (Sayers, 1950).

It is commonly accepted (Gaunt *et al.*, 1949, Harrison *et al.*, 1950) that the hyperkalemia of adrenal insufficiency is the result of adrenal retention of this ion and conversely that the hypokalemia seen in conditions where an excess of adrenal cortical hormone exists, are the result of an increased renal excretion of potassium (Darrow *et al.*, 1942, Sprague *et al.*, 1951). However, a decreased excretion of potassium during periods of adrenal insufficiency and increased excretion during prolonged treatment with adrenal cortical hormone has not consistently been shown either in the experimental animal or in patients (Perera *et al.*, 1949, Sartorius, 1951, Seldin *et al.*, 1951). Barnett *et al.* (1949) in careful balance studies found an increased excretion of potassium at the same time as the plasma level was elevated, during adrenal insufficiency. Administration of desoxycorticosterone corrected the high plasma potassium levels although no increase in the renal excretion of potassium occurred. From this, he postulated that

the effect of desoxycorticosterone is mediated through shifts of potassium between intra- and extracellular fluids. Others have reported that the administration of cortisone either in Addisonians or to patients with intact adrenals has not consistently caused an increased excretion of potassium (Perera *et al.*, 1949, Thorn *et al.*, 1951) although hypokalemia has frequently been reported (Baehr *et al.*, 1950, McEwen *et al.*, 1950, Sprague *et al.*, 1950). Harrison *et al.* (1939) report an increased urinary concentration of potassium following cortical hormone; however calculation of the total amount excreted in microeq./minute reveals that a significant increase in excretion did not always occur at a time when the plasma potassium level was decreasing. Our experiments do not confirm that the derangements of potassium metabolism in adrenal insufficiency are entirely the result of abnormalities in urinary excretion. From this, we infer that the influence of cortisone on potassium is only partially mediated through renal excretory mechanisms.

The finding that glomerular filtration rate and renal blood flow were restored toward normal during periods of adequate maintenance therapy are in agreement with the reports of others. The fact that these renal functions were not significantly altered within a two to three hour period following cortisone or desoxycorticosterone is consistent with the concept (Russel, 1950, Smith, 1951) that the functional decrease in filtration rate in adrenal insufficiency is, in part, secondary to the loss of extracellular fluid volume as well as a decreased plasma sodium concentration with a consequent derangement of renal hemodynamic factors. If this were true, it would be expected that the depressed filtration rate would not be increased, following cortisone administration, until the plasma electrolyte concentration and circulating fluid volume were restored toward normal. Such was the case in our experiments. Since our animals were not studied over a sufficiently long period of time to determine any irreversible pathological changes in renal functions, our experiments do not further any knowledge of the influence of cortisone or desoxycorticosterone on such changes.

SUMMARY

Cortisone administration in the adrenalectomized dog results in a marked decrease in the renal excretion of sodium. Prolonged administration of cortisone corrects the renal defect in sodium excretion and corrects the abnormal plasma sodium and potassium levels characteristic of adrenal insufficiency. During periods when adequate replacement therapy is maintained with cortisone in the adrenalectomized dog, the glomerular filtration rate and renal plasma flow are restored toward normal.

The effects of cortisone on the renal excretion of sodium have been com-

pared to the effects of desoxycorticosterone. It was found that both steroids affected sodium excretion in a similar manner. Administration of minimal doses of desoxycorticosterone and cortisone together decreased the renal sodium excretion by an amount that was of greater magnitude than that seen when each was given alone.

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INTRAVAGINAL ASSAY OF ESTROGENS

F. G. SULMAN

Department of Pharmacology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

ADVANTAGES OF THE INTRAVAGINAL METHOD

THE intravaginal Allen-Doisy test, though advocated by Berger (1935, 1937), Lyons & Templeton (1935/1936), Yerby (1937), Stadler & Lyons (1938), Freud (1939), Emmens (1941, 1947, 1950a,b), Muehlbock (1949), Biggers (1951), has hitherto been paid little attention in the literature and in practice. The reason for this is that in practical determinations its use always requires control with the international unit in general use, or with a standard weight unit for estradiol and estriol.

We feel, however, that the intravaginal Allen-Doisy test may become of decisive importance in the detection of estrogenic hormones in natural fluids, especially when these are available in relatively small quantities (cysts and normal blood), or when the estrogen is contained in the fluid in relatively low concentration, since by its use the injection of large toxic amounts of fluid or the performing of complicated extractions of normal urine or plant juices can be avoided. It should further be borne in mind that the subcutaneous assay cannot be very reliable, when a restricted quantity of material is available, since only a small number of animals can be used, whereas with the intravaginal Allen-Doisy test the accuracy may be highly enhanced by the use of a relatively unlimited number of animals.

The shortcomings of the Frank Test, a very important routine method for the early determination of fetal death by the assay of blood estrogens in pregnancy, can easily be overcome by its intravaginal modification: hitherto it could only be performed on unused castrated female mice, since these become anaphylactically sensitized by an injection of human serum and may die during a repeated assay.

Assays of estrogens in oily solution can also be carried out by the intravaginal method, thus avoiding the formation of oil cysts, encountered after repeated use of spayed animals for subcutaneous assays.

A further advantage of the intravaginal Allen-Doisy test is that it lasts only 2 to 2½ days, whereas the subcutaneous Allen-Doisy test lasts 4 days.

RESULTS PREVIOUSLY OBTAINED WITH THE INTRAVAGINAL METHOD

It is the general consensus that the intravaginal application of estrogens

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requires much smaller amounts for positive responses than do other methods. Irregularity of the results due to the vehicles used for the intravaginal instillation was a shortcoming described by most of the early authors. Thus, aqueous vehicles are active only in a very limited range of dosage, which is liable to permit resorption from the vaginal tract. Oily vehicles are better suited for rats, whereas the mouse vagina responds poorly to such preparations. The best vehicle so far described (Muehlbock, 1940 and Emmens, 1940/41), is the 50% aqueous glycerol solution. Recently, Biggers (1951) has proposed the employment of 1% aqueous egg albumin. We have found propyleneglycol and monoacetin similarly active in a 50% aqueous mixture, whereas carbitol was found less suitable because it may form precipitates, e.g. with human serum. The cited authors, using slightly different techniques, found almost corresponding values for the activities of the three natural estrogens, and their results are compared with our values in Table 1.

TABLE 1. INTRAVAGINAL ACTIVITY OF THE THREE NATURAL ESTROGENS

Hormones	Emmens 2 installations (gamma)	Muehlbock 3 instillations (gamma)	Sulman 5 instillations (gamma)	Enhancement of sensitivity obtained with 5 instillations as compared with 2 or 3	s.c./intravaginal ratio with 5 instillations
Estrone	0.0005	0.00025	0.0001	2.5– 5×	1,000
Estriol	0.001	0.00075	0.00005	15 – 20×	20,000
Estradiol	0.0005	*1940:* 0.0005 *1941:* 0.000005	0.000005	1 –100×	2,000

Emmens (1941) has shown that the natural estrogens are not proestrogens, i.e. that they are true, locally active estrogens which require no conversion in the body. The same holds for the stilbestrol derivatives. He further stressed the fact that esterification does not augment the estrogenic effect intravaginally, nor does it interfere with the performance of the test.

PRESENT TECHNIQUE

Our technique did not differ essentially from those used by Muehlbock and Emmens. Aqueous extracts were always administered after dilution (50%) with glycerol. The addition of glycerol doubled the estrogenic effect, thus making up exactly for the inconvenience of doubling the volume of fluid to be instilled into the vagina. The mouse vagina can hold fluid up to 0.02 cc. at a time if suitable animals are selected.

The small quantities of solution are best delivered by a micrometer syringe, such as the Agla micrometer syringe developed by the Wellcome Physiological Research Laboratories, Beckenham, Kent, England. It consists of a specially calibrated micrometer screw gauge attached to an all-glass syringe of narrow, uniform bore; to this a Record

needle of No. 1 gauge is attached, made up into a blunt cannula ½ cm long, which corresponds exactly to the length of the mouse vagina, thus avoiding any damage to the vagina. The aperture may be at the side or at the tip of the cannula. The plunger of the micrometer head impinges directly on the piston of the syringe, the piston being advanced by turning the head of the micrometer. The pitch of the micrometer screw is 0.5 mm.; the piston of the syringe is of such a diameter that two revolutions of the micrometer head eject 0.02 cc. (the maximal dose held by a mouse vagina). Since we administered every test solution 5 times, two full revolutions given 5 times corresponded to 0.1 cc. (maximal dose). The head of the micrometer is divided into 50 parts corresponding to 0.002 cc. solution. Hence the smallest dosage given by us in 5 instillations was $5 \times 0.002 = 0.001$ cc. The dose scale was as indicated in Table 2.

TABLE 2

Spayed female mouse	Test solution + glycerol cc. contained in 5 applications	Micrometer revolutions
1	0.0005 + 0.0005 = 0.001 cc.	5× 1/50
2	0.001 + 0.001 = 0.002 cc.	5× 2/50
3	0.0025 + 0.0025 = 0.005 cc.	5× 5/50
4	0.005 + 0.005 = 0.01 cc.	5× 10/50
5	0.01 + 0.01 = 0.02 cc.	5× 20/50
6	0.025 + 0.025 = 0.05 cc.	5× 50/50
7	0.05 + 0.05 = 0.1 cc.	5×100/50

The micrometer syringe was conveniently attached to a stand at a 45° angle, as shown in Fig. 1, thus avoiding the release of more fluid during the intervals between the 5 instillations. The animal is firmly gripped with the left hand, the thumb and 2nd finger holding the wrinkled neck skin, the 4th and 5th fingers holding the tail, and the bent 3rd finger pushing the body of the mouse (or rat) forward into a more or less semi-arched position. In this position the animal's vagina is gently pulled over the blunt tip of the cannula. With the right hand the micrometer can then be reliably manipulated. This method permits well controlled intravaginal administration without any assistance.

Although the zero hour was taken as a rule, it is not necessary if the animals are rested for sufficient time after a positive test. Staining and scoring details are similar to those in the subcutaneous Allen-Doisy method. It should, however, be stressed that the intravaginal method induces a much higher percentage of proestrus and metestrus responses than other methods, and these should be considered positive only if the number of leucocytes does not exceed the number of epithelial cells in the microscopic field.

Smears were positive after the administration of estriol as early as 36 hours after instillation, whereas with estrone the effect appeared only after 48 hours, and with estradiol it was sometimes delayed until 60 hours.

Priming of the animals was found necessary, as stressed by Emmens (1950). Repeated use of the animals not only increased their initial sensitivity, but also led to much more uniform and constant reactions in further tests.

The following is a report on the results obtained with a total of 1500 spayed female mice.

EXPERIMENTAL

1. *Urine estrogens*

Female urine in the ovulatory and the premenstrual stage gave positive vaginal reactions when diluted with glycerine āā. During the other stages

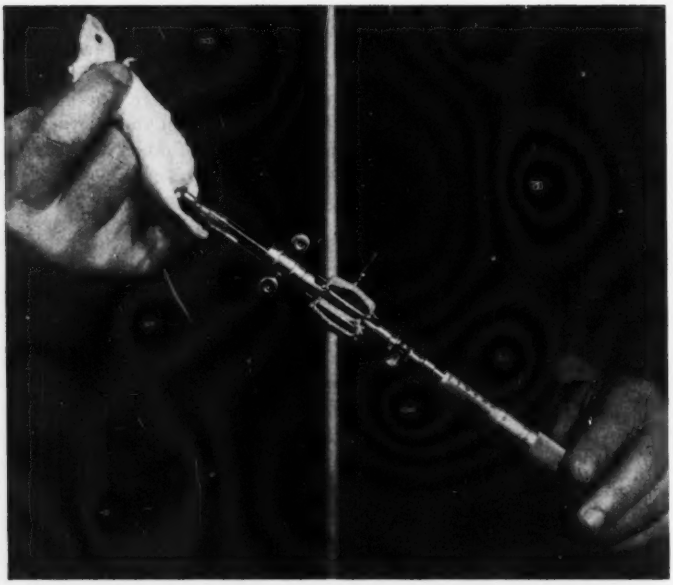

FIG. 1. Technique of intravaginal assay of estrogenic hormones.

The standard schedule was as follows:

1st day: zero hour smear followed by 3 instillations;
2nd day: 2 instillations followed by 1 smear in the evening; with high doses this 36 hour smear was sometimes positive;
3rd day: 48 and 60 hour smears. Further smears were not necessary with the 5 instillations method, but are necessary with methods involving 2 or 3 instillations.

the urine had to be concentrated 10 fold. To 10 cc. of urine 1 cc. glycerol was added and the mixture was heated until the water had completely evaporated; 1 cc. of aqua dest. was then added, and the estrogens were thus dissolved in a 2 cc. glycerol-water āā solution.

When male urine was used this concentration was sufficient to elicit a positive reaction in only a certain proportion of the cases. Hence we used double the concentration, i.e. we evaporated 20 cc. of urine in the presence of 1 cc. of glycerol, later adding 1 cc. of aqua dest.

In cases of pregnancy and chorionepithelioma the concentration was of course unnecessary. It was required, however, in most cases of mole, since estrone excretion in mole may be extremely low.

Assay values obtained by the intravaginal method were multiplied by the factor 400 to be converted into I.U.

2. *Blood estrogens*

Blood was either studied as whole blood or was divided into two fractions, corpuscles and plasma or serum. When we studied corpuscles and plasma separately, a greater concentration of estrogens was found in the plasma.[1] Positive values without concentrating were obtained only at the ovulatory and premenstrual phases. Female blood at other stages and male blood had to be concentrated.

For concentrating 10 x, we started from plasma or serum. To 10 cc. plasma or serum 40 cc. acetone were added, the precipitated protein was removed by centrifugation, washed once with a little ether, and the ether and acetone were then pooled and evaporated. The residue was dissolved in 1 cc. glycerol+1 cc. aqua dest. by heating for 5 minutes in a water bath, at 100° C.

The intravaginal assay of pregnancy serum without any previous concentration is a most important advantage, since it permits the performance of the Frank test on a large number of animals with less than 1 cc. of serum.[2] Furthermore, it permits the repeated use of the assay animals without fear of anaphylactic reaction as encountered when subcutaneous injections are given.[3] The same holds true for the assay of aspirated follicular fluids. Our results with this technique will be published separately.

Assay values for serum estrogens obtained with the intravaginal method were multiplied by the factor 400 to be converted into I.U.

DISCUSSION

The advantages of the intravaginal as compared with the subcutaneous method are manifold, especially in the assay of naturally occurring estrogens. The subcutaneous technique permits the injection of no more than 5 cc. blood or urine into mice, viz. the determination of as many as 200 MU estrogen per liter fluid. Smaller amounts cannot be determined without concentrating the fluid. This is, however, not always possible with blood, because of the limited quantity available. With the intravaginal method 1 vaginal unit may be assayed in 0.1 cc. fluid. Since the conversion factor from the vaginal unit to the subcutaneous unit is, 400 for blood and urine, the intravaginal technique permits the determination of 1/400 I.U. in 0.1 cc. fluid i.e. 25 I.U. per liter blood or urine. This threshold concentration is within the range of the normal estrogen content of serum or urine and

[1] This is in contradiction to the finding of Albrieux (1941).

[2] Forty cc. of clotted blood was hitherto required.

[3] If animals repeatedly treated intravaginally with human serum receive a subcutaneous injection of human serum, a high percentage of them also die, since the intravaginal priming induces anaphylaxis following subcutaneous injection.

is easily obtained at peak days (during ovulation, antemenstrual phase). Hence, the intravaginal technique can detect stages of slight hyperestrinism which would have escaped our attention with the routine subcutaneous technique (hyperestrinism in liver cirrhosis, B-avitaminosis, etc.).

The intravaginal technique, if combined with simple concentration methods, as described above, further allows us to study conditions neglected until now: hypestrinism and anestrinism, which require investigation in view of the fact that body estrogens have at least 3 sources: ovaries, adrenals, nutrition. A priori, it seems improbable that all 3 sources should fail simultaneously, but the possibility must be borne in mind.

As to the conversion of the vaginal unit into the subcutaneous and international unit, it is difficult to explain the marked differences between conversion factors which are for blood and urine 400, estrone in aqueous solution 1000, estradiol in aqueous solution 2000, estriol in aqueous solution 20,000.

This variation may depend upon poor resorption of blood and urine estrogens via the intravaginal route. In the case of estriol, it may be connected with the great variations in its effect depending upon the method of administration, an oily solution being .10 times more active than an aqueous one (Zondek & Sulman 1951).

SUMMARY

A technique has been described for the intravaginal assay of minute amounts of estrogens by the intravaginal route. The smallest amounts of estrogen detectable by this method in mice are 0.0001 gamma estrone, 0.00005 gamma estriol, 0.000005 gamma estradiol.

Working with pure estrogens, the factor for converting the vaginal unit into the subcutaneous unit is 1000 for estrone, 20,000 for estriol, and 2000 for estradiol. Mixtures of these estrogens occurring in natural fluids together with proteins and minerals yield much lower factors of conversion: 400 for blood (serum or plasma) and for urine.

Aqueous assay fluids are preferably applied to the vagina as a mixture with equal parts of glycerol or propyleneglycol or monoacetin. The consequent doubling of the volume is compensated for by a twofold titer of the estrogens in these solutions. The minimal dosage applicable to the vagina was found to be $5 \times 0.0002 = 0.001$ cc., the maximal dosage retained is $5 \times 0.02 = 0.1$ cc. Regularity of the results is in accord with the experience gathered from subcutaneous injections. Priming of the animals considerably increases their sensitivity and the regularity of the results.

The advantages of the intravaginal assay are:

1) Possibility of detecting minimal amounts of estrogens, thus permitting the diagnosis of states of hypestrinism and anestrinism;

2) Exact assay of fluids available in relatively small quantities e.g. cysts and blood, with a relatively unlimited number of animals;
3) Assay of estrogens in toxic fluids without extraction (plant juices, decomposed urine);
4) Possibility of repeated serum administration in animals primed by the assay of blood estrogens (Frank test) for the determination of fetal death, without danger of anaphylactic death of the animals under test;
5) Repeated use of primed animals for the assay of estrogens in oily solutions without the formation of subcutaneous oil cysts;
6) Clear-cut results may be obtained within 2 to $2\frac{1}{2}$ days, instead of within 4 days.

Acknowledgments

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THE EFFECT OF EPINEPHRINE AND THYROTROPIN ON THYROID FUNCTION IN RATS

A. L. BOTKIN AND H. JENSEN

From the Army Medical Research Laboratory, Fort Knox, Kentucky

INTRODUCTION

WHILE the action of thyrotropic hormone on the thyroid gland is well known, the role of epinephrine is not. It has been claimed (Reiss 1949) that epinephrine administration increases the uptake of radioactive iodine by the thyroid. However, Soffer and associates (1949) reported that treatment with epinephrine decreases the uptake of radioactive iodine. These investigators also observed that the administration of epinephrine resulted in an increased secretion of thyrotropin by the pituitary.

In view of these discrepancies it was felt desirable to investigate further the effects of epinephrine administration on thyroid function after short time intervals. Of particular interest in this connection was an interpretation of the significance of the radioiodine content of the thyroid, or "uptake" as it has frequently been called. Another point under consideration was the validity of the prevailing concept of the thyroid gland being a sluggish organ, slow in responsiveness to stimuli.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain (weighing 190–250 grams), maintained on Purina laboratory chow and tap water, were used. Twenty-four hours before sacrifice, at which time they were deprived of food but allowed water *ad lib*, each rat was injected intraperitoneally with 1.0 ml. of radioiodine solution containing approximately 5 microcuries of carrier-free radioactive iodine (I^{131})[1] made up to volume in phosphate buffer of pH 7.4 (Krebs and Henseleit 1932).

The animal was sacrificed by a sharp blow at the base of the skull and exsanguinated via a thoracic incision. The thyroid gland was rapidly removed, weighed, and analyzed for inorganic and organic radioiodine content using a modification of the method of Morton and Chaikoff (1943).

For serum studies, blood, drawn by cardiac puncture just prior to sacrifice, was allowed to clot and serum was obtained by centrifugation. One aliquot of serum was dried and counted as total serum radioiodine content. Another aliquot was precipitated

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[1] The radioactive iodine (I^{131}) used in this investigation was supplied by the Oak Ridge National Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

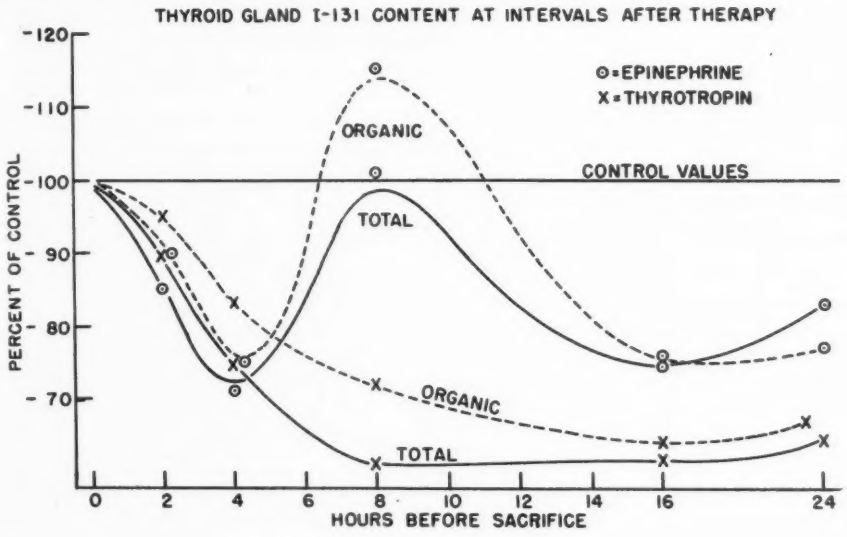
THYROID GLAND I-131 CONTENT AT INTERVALS AFTER THERAPY
⊙=EPINEPHRINE
X=THYROTROPIN
ORGANIC
CONTROL VALUES
TOTAL
ORGANIC
TOTAL
PERCENT OF CONTROL
120
110
100
90
80
70
0 2 4 6 8 10 12 14 16 24
HOURS BEFORE SACRIFICE

FIG. 1.

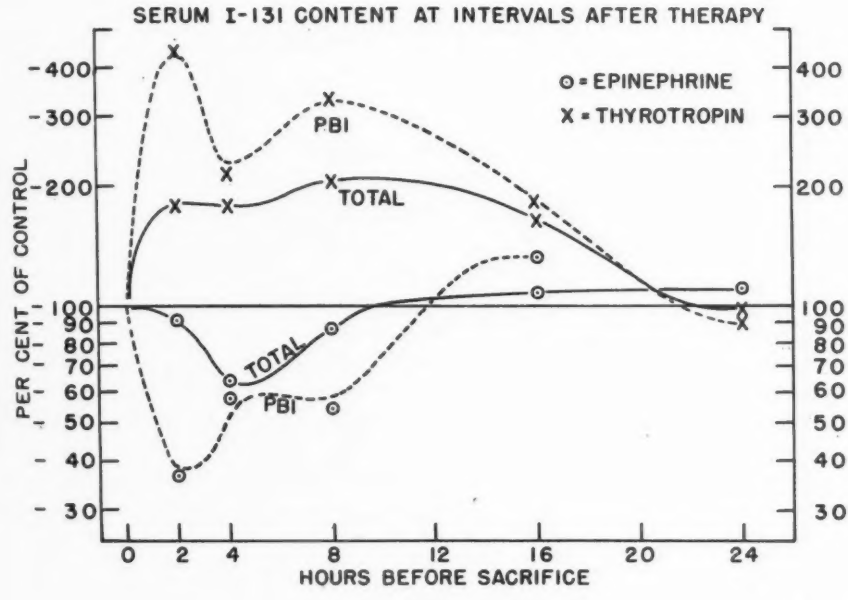
SERUM I-131 CONTENT AT INTERVALS AFTER THERAPY
⊙=EPINEPHRINE
X=THYROTROPIN
PBI
TOTAL
TOTAL
PBI
PER CENT OF CONTROL
400
300
200
100
90
80
70
60
50
40
30
0 2 4 8 12 16 20 24
HOURS BEFORE SACRIFICE

FIG. 2.

with trichloracetic acid and the precipitate was dissolved in 20% NaOH, dried and counted as the serum protein-bound radioiodine ("PBI^{131}").

All hormone administrations were by the intraperitoneal route. The following preparations and dosages were used:

1. Epinephrine;[2] 0.1 mg. per rat in 0.1 ml. saline solution.
2. Thyrotropic hormone[3] ("Thyrotropin," containing approximately 4 Junkmann-Schoeller units per mg., 6 mg. per ml. in saline brought to pH 7.5 with NaOH); 6 mg. per rat in 1.0 ml. of solution.

Three groups of animals were run simultaneously at each time period, the first (4 animals) receiving saline (controls), the second (6 or 8 animals) 0.1 mg. of epinephrine, and the third (6 or 8 animals) 6.0 mg. of thyrotropin ("TSH"). The groups were injected at intervals of 2, 4, 8, 16, and 24 hours before sacrifice.

All values for radioiodine content of the thyroid gland and serum were obtained as per cent of the injected dose. The values for the control animals were considered as 100 in determining the deviations produced by the experimental procedures. The average findings for each group are plotted in Figures 1 and 2.

RESULTS

In the gland content studies (Figure 1), it was found that by 4 hours after the administration of either TSH or epinephrine both total and organic values were lowered to approximately the same degree. By 8 hours, however, the glands of the epinephrine injected animals showed a return to normal, followed by a less severe drop. This second lowering might have been due to a "rebound" effect. The glands of the TSH injected animals, on the other hand, continued to show a decrease in their radioiodine content to a minimum average of about 60% of the control values at 8 hours. This level remained fairly constant with a possible drift back toward normal apparent by 24 hours.

In Figure 2, the plotting of the serum total I^{131} and PBI^{131} values was of necessity reproduced on a log scale to include the magnitude of the resulting variations. The TSH injected animals showed within 2 hours a sharp rise in total serum radioiodine content to approximately 200% *above* control values. This level stayed relatively constant until 16 hours after which time it gradually returned to a normal value at 24 hours.

The main component of this total increase consisted of the PBI^{131} fraction, which rose to its maximum of 441% *above* control levels at 2 hours. The results found in the epinephrine treated animals were opposite in effect to those seen in the TSH treated groups. Here the total serum radioiodine content dropped to a minimum of 63% by 4 hours and by 8 hours was practically normal. Again the main alteration was due to the PBI^{131} component which fell to 36% in 2 hours and by 12–16 hours had returned to slightly above normal.

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[2] Upjohn buffered tablets made up to 1.0 mg. per ml.

[3] Obtained through the courtesy of Armour and Company, Chicago, Illinois.

DISCUSSION

The results obtained may be explained on the basis of the comparative mechanisms involved. In the case of TSH administration the mechanism is a stimulation of the thyroid gland resulting in a "flushing-out" of hormone from the gland with a consequent rise in serum concentration of hormonal radioiodine. This may explain the drop in gland content. A concurrent increased turnover (or conversion) rate as well as an increased uptake of I^{131} would tend to keep the ratio of organic to inorganic I^{131} components constant. The serum picture follows the expected pattern of an abrupt rise in serum concentration of hormonal radioiodine.

By contrast, the metabolic effects of epinephrine administration on the peripheral tissues may cause an increased demand for thyroid hormone. This demand produces an immediate utilization and a resulting decrease of the available serum hormone. This initiates a release of pituitary TSH simulating the same initial gland picture as that seen after TSH injection and tending to restore the thyroxine blood level to normal. The serum level remains lower than the control values due to the increased hormone utilization by peripheral tissues. In addition it is also possible that epinephrine may have a direct effect on the pituitary gland, causing a release of TSH.

The picture of thyroid-pituitary relationship seems to be analogous to that existing in the pituitary-adrenal cortex axis. Here also an increased secretion of pituitary adrenocorticotropic hormone (ACTH) may first be preceded by an increased peripheral utilization of the adrenal cortex principles followed by a decrease in blood level of these hormones, causing the release of ACTH from the pituitary, etc. Another interesting comparison between thyroid and adrenal cortex can be seen in the relation of gland composition to functional activity. A low cholesterol content of the adrenal gland may, under certain conditions, indicate a stimulation of this endocrine organ with a resultant outpouring of its hormones. A similar relationship may also hold true for the iodine content and functional activity of the thyroid gland. Money, Kirschner, Kraintz, Merrill and Rawson (1950) assumed that a low gland-iodine content is synonymous with a low "uptake" or collection of iodine by the gland. It is apparent from the results presented here that there is not always a proportional relationship between "content" and "function" nor necessarily between "content" and "uptake." In an analysis of thyroid function, therefore, iodine "content" must be considered as a resultant of production and release of hormone as well as uptake of iodine.

It can also be seen from the above results that the thyroid gland responds quickly to either epinephrine or TSH administration, for within 2 hours, particularly as seen in the serum PBI^{131} studies, the degree of production and release of thyroxine has varied significantly from the normal.

Hamolsky, Gierlach and Jensen (1951) observed an almost immediate response of the thyroid of the rat when subjected to "tourniquet shock." Thus, the concept of the thyroid gland being a sluggish organ, slow in responding to stimulation, does not seem to be valid.

SUMMARY

There was an early decrease in gland content of radioactive iodine (I^{131}) after either epinephrine or thyrotropin administration. The serum content after thyrotropin injection was increased (particularly the protein-bound component) due to acceleration of the production and release of thyroxine by the thyroid gland. In the epinephrine treated rats, however, the serum content was decreased. This is interpreted as being due to the increased peripheral utilization of thyroxine. The decreased serum thyroxine level probably causes an increased pituitary release of thyrotropin and therefore produces the same effect on the thyroid gland as thyrotropin administration.

The thyroid gland has been found to be quickly responsive to TSH and epinephrine administration.

A decreased content of radioiodine in the thyroid is not necessarily indicative of decreased functional activity. This suggests a more cautious use of the term "uptake."

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SOME STUDIES ON THE OXIDATIVE METABOLISM OF THE ADRENAL GLAND

T. L. SOURKES AND P. HENEAGE

From the Merck Institute for Therapeutic Research, Rahway, N. J.

NUMEROUS studies have served to elucidate the physiology and biochemistry of the adrenal medulla and cortex, but at the same time have paid scant attention to the metabolic pattern underlying the endocrine phenomena of these tissues. Because of the lack of information on the nature and scope of intermediary metabolism of the adrenal, it was deemed important to initiate such an investigation. The present paper describes various reactions occurring in the tricarboxylic acid cycle and in the metabolism of a number of amino acids and other compounds, as carried out by isotonic homogenates and by slices of the adrenal gland. Some of these reactions have been studied in several tissues for comparative purposes.

METHODS

Preparation of Animal Tissues: Most of this work was done with beef adrenals, which were obtained at a nearby slaughterhouse. The organs were dissected out within 30 minutes or less of the death of the animals, and then kept in ice-cold saline until used in experiments one to 4 hours later. In initial experiments tissues frozen on dry ice were used. Oxidative activity was often retained by adrenals preserved in this manner for at least several hours, in contrast to the rapid loss of activity by beef heart muscle, kidney cortex and liver kept under identical conditions. However, this activity was too inconsistently retained to warrant using such frozen glands in a standard procedure.

Guinea pigs and rats were killed by stunning. The organs required were quickly dissected out and used immediately.

Homogenates were prepared in isotonic KCl with the Potter-Elvehjem glass homogenizer. 20% homogenates were used, except where otherwise indicated. Tissue slices 0.5 mm. thick were cut conveniently with a Stadie-Riggs microtome. The section was always made transverse to the axis of one limb of the adrenal so that representative portions of both cortex and medulla were obtained.

Reaction Mixtures: For the study of oxidations in homogenates a standard reaction mixture was used, made up of the following ingredients (final concentrations given): K phosphate buffer, pH 7.4 (0.007 M); KCl (0.115M); adenylic acid (0.0013M); $MgCl_2$ (0.0033M); substrate (0.0166M). The adenylic acid and the substrates were neutralized with KOH to pH 7.4 before addition to the flasks. 0.6 ml. of homogenate was added to give a final volume of 3.0 ml., except where otherwise noted. Because of its incompatibility with the buffer the magnesium salt was always added as the last item, i.e. after the tissue had been pipetted in.

When tissue slices were used the suspending medium was the buffer of Krebs and

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Eggleston (1940). The additions to the flasks consisted of 2.5 ml. of this buffer together with 0.5 ml. of M/10 substrate (neutralized sodium salt).

Standard manometric techniques were employed for the measurement of oxygen uptake (Umbreit, Burris and Stauffer, 1949). All incubations were performed at 37°. All tabulated data represent means of at least three determinations, except those of Table 4 (two determinations).

RESULTS

1. *Beef adrenal homogenates*

The oxidation of a variety of substrates by beef adrenal homogenates is illustrated in Figure 1. The rates of oxidation were relatively steady for 60 minutes or more. While the Krebs' cycle intermediates were rapidly metabolized in this preparation, other substrates were poorly handled. The following amino acids gave no oxygen uptake when incubated with beef adrenal homogenates in pyrophosphate buffer, pH 8.3 (the optimal pH): glycine, dl-alanine, dl-methionine, dl-tryptophan, dl-serine, dl-aspartic acid. Acetate, butyrate and acetoacetate were not oxidized in the standard system. Among the amino acids tested in the standard system serine, threonine and aspartic acid did not increase the oxygen uptake of the homogenate. The l- and d-amino acid oxidases are present in but very low concentrations as judged from the rates of oxidation of typical substrates for these two enzymes (see curves for l- and dl-methionine). In the case of glutamic acid, while its rate of oxidation was greater than that of any of the other amino acids tested it was still well below that of its own oxidation products, namely α-ketoglutaric acid.

In a number of experiments it was noted that pyruvate was oxidized at a rather slow rate. This is in contrast to the high rate observed when tissue slices were used (see below), and suggested that the homogenate reaction mixture was incomplete even after the addition of the usual cofactors. Since the entrance of pyruvate (or acetate) into the Krebs' cycle is accomplished with the participation of oxaloacetic acid it was thought that the addition of the latter compound to the reaction mixture might stimulate the oxidation of pyruvate and acetate. Only traces of oxaloacetic acid (or some other acid which can form oxaloacetic acid in the homogenate) would be required since the compound is regenerated cyclically. This experiment was carried out with the results shown in Table 1. It is clear that small amounts of the C_4-dicarboxylic acids stimulate appreciably the oxidation of pyruvate and acetate in adrenal gland homogenates. Data similar to those for acetate were also obtained for the catalysis of butyrate oxidation by traces of malic acid.

2. *Comparison of cortex and medulla*

Since portions of both cortex and medulla were included in the homogenates used in the above experiments an attempt was made to determine

what proportion of the total oxygen uptake each was contributing. Beef cortex and medulla were separated from one another by dissection immediately after removal of the gland from the animal, and were transported

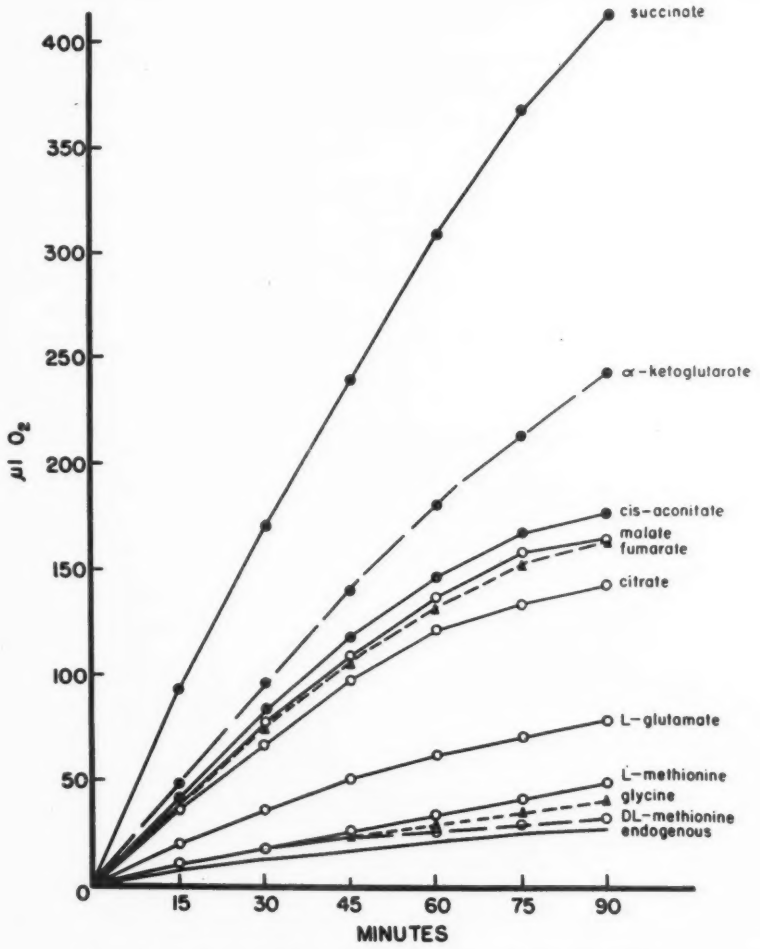

FIG. 1. Oxygen uptake by beef adrenal gland homogenates in the presence of various substrates.

to the laboratory where they were homogenized and used at once. The results (Table 2) indicate that the ability of the medulla to oxidize common metabolic intermediates is well below that of the cortex.

TABLE 1. OXIDATION OF PYRUVIC AND ACETIC ACIDS BY BEEF ADRENAL HOMOGENATES

Microliters of oxygen uptake per 100 mg. wet weight of tissue in 60 min. (corrected for endogenous respiration). Final concentrations: Pyruvate, 0.0167 M; acetate, 0.0167 M; C_4-dicarboxylic acid, 3.3×10^{-4} M. Each flask contained 120 mg. wet weight of tissue. Other details in text

Experiment No.	Substrates	O_2 uptake (microliters)
1A	Pyruvate	42
	Oxaloacetate	41
	Pyruvate + Oxaloacetate	130
1B	Pyruvate	42
	Fumarate	80
	Pyruvate + Fumarate	137
2	Pyruvate	17
	l-Malate	28
	Pyruvate + l-Malate	63
3	Acetate	3
	l-Malate	113
	Acetate + l-Malate	151

TABLE 2. COMPARISON OF BEEF ADRENAL CORTEX AND MEDULLA IN RESPECT TO OXIDATION OF INTERMEDIATES OF THE TRICARBOXYLIC ACID CYCLE

Q_{O_2} = microliters of oxygen consumed/hr./mg. dry matter (based on uptake in first 45 minutes of incubation). 20% isotonic KCl homogenates used (120 mg. of tissue per flask)

Substrate	Cortex	Medulla
None	1.1	0.6
Citrate	6.0	0.8
Cis-Aconitate	6.7	2.3
α-Ketoglutarate	8.6	2.4
Succinate	13.6	6.5
Fumarate	6.2	1.2
l-Malate	7.5	1.7

TABLE 3. SPECIES VARIATIONS IN THE OXIDATION OF SUBSTRATES BY ADRENAL GLAND HOMOGENATES

Q_{O_2} = microliters of O_2 consumed/mg. dry matter/hr., based on oxygen uptake in the initial 15-min. period following equilibration. (Respective series of flasks contained: beef, 120 mg.; guinea pig, 96 mg.; rat, 35 mg. wet weight of tissue. In the rat adrenal series, all additions to the flasks were reduced proportionately to give a total volume of 1.0 ml.)

Substrate	Beef	Guinea pig	Rat
None	1.5	1.7	1.8
Citrate	7.7	11.4	8.2
Cis-Aconitate	9.2	—	7.3
α-Ketoglutarate	10.3	8.9	12.2
Succinate	19.7	11.1	13.3
Fumarate	9.0	4.3	4.9
l-Malate	8.8	—	4.2

3. *Species variation*

Experience in manometric studies with the adrenals of species other than beef indicated a basic pattern with no marked species differences. Table 3 shows the initial rates of oxidation of a number of substrates by beef, guinea pig and rat adrenal homogenates.

While the figures for a given substrate were more or less of the same order, guinea pig adrenals oxidized citrate more rapidly than did the other adrenals. On the other hand the initial rates of oxidation of the C_4-dicarboxylic acids were greatest with the beef adrenals.

TABLE 4. COMPARISON OF OXIDATION IN HOMOGENATES OF (A) BEEF AND (B) GUINEA PIG TISSUES

Figures represent microliters of O_2 uptake/100 mg. wet tissue/hr. (120 mg. of beef tissues or 96 mg. of guinea pig tissues used per flask)

Substrate	Adrenal	Beef Kidney	Heart
None	16	28	57
Citrate	94	57	146
Cis-aconitate	106	75	158
α-Ketoglutarate	140	214	221
Succinate	269	280	263
Fumarate	113	183	133
l-Malate	109	126	91

Substrate	Adrenal	Guinea pig Kidney	Liver
None	36	161	91
Citrate	245	285	232
α-Ketoglutarate	187	248	227
Succinate	250	364	328
Fumarate	115	240	174

4. *Metabolic activity of the adrenals compared to other tissues*

In order to clarify the relative activity of adrenal homogenates two series of experiments were carried out comparing their performance in parallel tests with homogenates of other beef tissues. Homogenates of beef heart (ventricle), kidney cortex and adrenal were studied in two experiments with the average figures obtained shown in Table 4. The adrenal showed the lowest endogenous rate of respiration but this rate was nevertheless capable of very marked stimulation by all the substrates tested, especially succinic acid. One feature of adrenal metabolism is of interest, namely the quite similar rates of oxidation of the added substrates (apart from succinate whose requirement of co-factors for oxidation is less complex than in the cases of the other substrates). In the other two tissues the rates are much more diverse.

Guinea pig adrenal, liver and kidney (whole tissue) were compared using 16% isotonic homogenates (Table 4). In this species the adrenal also dis-

played a low endogenous rate of oxidation. Citrate oxidation proceeded rapidly, fumarate slowly. Succinate and fumarate, respectively, both stimulated respiration to about the same extent in all three tissues (i.e. considering oxygen uptake after deducting the endogenous rate).

5. *Beef adrenal slices*

While the homogenate technique is very useful in the study of cellular oxidations and other reactions it must be kept in mind that this preparation consists of disrupted cells. In the process of homogenization the "normal" metabolic behavior of the tissue may be altered because a) the cellular membrane is destroyed with the consequent release of intra-cellular struc-

TABLE 5. COMPARISON OF RATE OF OXIDATION OF SUBSTRATES BY BEEF ADRENAL HOMOGENATES AND SLICES

Substrate	Homogenate	Slices
Endogenous	1.2[1]	2.0[1]
Citrate	6.9	3.6
Cis-aconitate	8.3	3.8
α-Ketoglutarate	9.9	5.8
Succinate	16.8	10.7
Fumarate	7.5	4.3
l-Malate	7.6	4.8
Pyruvate	2.2	4.9
Acetate	1.2	3.3
Butyrate	1.2	4.8
l-Ascorbate	4.0	9.4
l-Glutamate	3.5	4.3

[1] Q_{O_2} = microliters of oxygen consumed per hour per mg. dry weight (based on 45 minutes uptake).

tures and alteration of their spatial relationships; and b) all constituents of the cell are diluted, particularly some of the co-factors essential to many enzymatic reactions. In order to learn how closely the reactions of the intact cells are reproduced by the homogenized tissue, beef adrenal gland slices were studied with the same substrates used for the homogenates (section 1). In order to permit comparison of slices and homogenates Q_{O_2} values have been calculated (Table 5).

The adrenal homogenates appear to oxidize the Krebs' cycle intermediates at considerably faster rates than do the slices. The reverse is true in the cases of pyruvate and acetate, whose oxidation by homogenized tissue can be catalyzed by trace amounts of a C_4-dicarboxylic acid, as shown in section 1. These two compounds, as well as butyrate, are oxidized by adrenal slices with apparent ease, presumably because of the continuous supply of the dicarboxylic acids in the intact cells. Ascorbic acid is oxidized by both preparations.

6. *The effect of steroids* in vitro *on adrenal metabolism*

That physiologically active steroids may exert definite *in vitro* effects on tissue metabolism has been established by previous authors. Gordon and Elliott (1947) found that testosterone and certain other steroids depressed the oxidation of glucose by rat brain slices. They later showed (Eisenberg, Gordon and Elliott, 1949) that suspensions of testosterone likewise inhibited the uptake of oxygen by rat liver and skeletal muscle slices. The specific nature of the inhibition was pointed out, since several other steroids did not inhibit. Moreover the inhibitory steroids differed in their action quantitatively. The study by Eisenberg *et al.* (1949) indi-

TABLE 6. EFFECT OF CORTISONE AND DESOXYCORTICOSTERONE *in vitro* ON OXIDATIONS IN BEEF ADRENAL, KIDNEY AND HEART HOMOGENATES

(Figures represent per cent changes in 45 min. O_2 consumption above (+) or below (−) control value observed with the respective substrates.)

Tissue	Adrenal		Kidney		Heart	
Steroid	E	DOC	E	DOC	E	DOC
Substrate						
Endogenous	−15	−32	−28	−45	−29	−73
Citrate	−11	−70	−10	−70	+ 6	−60
Cis-Aconitate	−40	−64	−15	−65	0	−60
α-Ketoglutarate	−35	−50	+20	−70	+30	−56
Succinate	− 9	+ 9	−10	+16	+10	+ 5
Fumarate	−55	−50	−65	0	+ 4	−60
l-Malate	−30	−50	+30	−60	—	—

cated that the site of steroid inhibition was not the cytochrome system. Hayano, Schiller and Dorfman (1950) observed inhibition of oxgyen consumption by rat liver, brain and kidney slices when various steroids were added *in vitro*. Among the compounds tested, desoxycorticosterone (DOC) was the most active. DOC also inhibited oxygen uptake by whole brain homogenate and by washed brain homogenate in the presence of substrates. In a second paper (Hayano, Dorfman and Yamada, 1950) they reported the inhibition of d-amino acid oxidase by DOC due to combination of the steroid with the apoenzyme. These marked effects prompted us to determine whether cortical steroids might likewise have *in vitro* actions on adrenal tissue metabolism.

To study the problem the Warburg flasks were prepared in the usual manner but in addition 1 mg. of the crystalline steroid was supplied in the total volume of 3.0 ml. (Cortisone is soluble in water to the extent of 27 mg. per 100 ml. at 25°; DOC is less soluble.) In preliminary experiments it was found that both cortisone (E) and DOC altered the rates of oxidation markedly in the cases of certain substrates. Furthermore it was noted that when the oxidation of an added substrate was inhibited by the steroid,

the degree of inhibition frequently increased to a maximum in about 45 minutes and then remained more or less constant. This plateau, representing the full effect of the steroid, is possibly the result of maximum contact between steroid and enzyme achievable under the conditions of the experiment, one such condition being the rate of solubilization of the steroid. The results with E and DOC added to adrenal homogenates are given in Table 6.

It will be seen that both steroids inhibit the oxidation of the substrates tested, and that DOC is the more potent inhibitor. The case of succinic acid is exceptional in that the effects of E and DOC are minimal and of little apparent significance.

Similar experiments were than carried out on beef kidney cortex and heart (ventricular) muscle in order to determine whether this pattern of *in vitro* effects on adrenal homogenates applied generally or whether the tissues exhibited a specificity in their reaction to the steroids. It is clear from the data of Table 6 that the three tissues were affected differently. For instance, while the endogenous respiration of heart muscle was reduced by E, this steroid increased the rate of oxidation of some added substrates and did not affect others. When supplied to kidney homogenates E increased the rate of oxidation of α-ketoglutaric and malic acids but inhibited in the case of other substrates. The predominant action of DOC was to depress the oxidation of substrates tested except succinic acid whose oxidation increased somewhat in the presence of this steroid. In addition DOC had no apparent effect on fumarate oxidation in kidney homogenates. The marked inhibition of endogenous respiration of cardiac muscle by DOC may be noted.

Beef adrenal slices were also tested with the same substrates as used for homogenates to determine whether E and DOC would exert similar *in vitro* effects. Both steroids stimulated the oxidation of citric and succinic acids; DOC also increased the oxidation of fumarate. Other oxidations (including endogenous respiration) were inhibited as in the homogenates but not to the same extent.

DISCUSSION

These studies show that as far as the oxidation of intermediates of the Krebs' tricarboxylic acid cycle are concerned the adrenal gland is not qualitatively different from a number of other tissues examined. However the adrenal seems to have a small complement of the enzymes oxidizing amino acids.

The low Q_{O_2} values obtained with homogenates of the adrenal medulla as compared with those prepared from the cortex indicate a minor role for the medulla in oxidations performed by beef adrenal homogenates. This

is pointed up further by the fact that in samples of the glands collected for use in this work the medulla made up only 20–30% of the fresh weight of the organ. Whether these relatively low rates are also true of the medulla *in situ* is, of course, not certain. That large differences may exist between cortex and medulla as seen in Table 2 should not be surprising, since these two tissues are embryologically, histologically and physiologically distinct, and could be expected to exhibit differences just as may spatially separate organs. Separation of the two parts of the adrenal was not attempted with guinea pig and rat glands. It is probable, however, that the medullae of these species also make only a small contribution to the respective Q_{O_2} values of the whole adrenal since guinea pig and rat medullae constitute only a small part of the gland (Hartmann and Brownell, 1949).

A comparison of adrenal homogenates and slices revealed some marked differences in the rate of oxidation of substrates. The intermediates of the tricarboxylic acid cycle were metabolized more rapidly in the homogenates than by the slices. It is possible that rates in slice experiments are limited by diffusion through the cell membrane. Other mechanisms are undoubtedly operating in the intact cell to govern the rate of metabolism, for respiration of adrenal slices in the absence of added substrate was considerably higher than that of the homogenates. Moreover acetate and butyrate, two substrates which are not oxidized by homogenized tissue unless catalytic amounts of C_4-dicarboxylic acids are supplied, were metabolized by slices as readily as were a number of Krebs' cycle intermediates.

The significance of the *in vitro* effects of E and DOC on adrenal metabolism is as yet difficult to assess. The actions of these steroids do not appear to be merely physical effects due to their surface activity and lipid solubility properties. In unpublished experiments we have obtained no effect by adding cholesterol to the homogenates, a steroid which would appear to meet purely physical requirements for activity. Moreover, as pointed out above, others have also found quantitative differences in the action of steroids *in vitro* and have reported on steroids which do not affect various *in vitro* metabolic reactions. The *in vitro* effects of E and DOC observed here on a series of comparable oxidative reactions occurring in beef adrenal, heart and kidney (Table 6) strengthen the view that the steroids may exert specific actions at one or more points in the enzyme systems catalyzing these oxidations.

SUMMARY

The intermediary oxidative metabolism of the adrenal gland has been studied by means of the homogenate and tissue slice technique. Intermediates of the tricarboxylic acid cycle and of fat oxidation (acetate, butyrate) were metabolized by both types of preparation, as were certain amino acids

(glutamic acid, methionine, glycine). Differences in the rate of oxidation of substrates by homogenates and slices were noted and the possible reasons for these differences were discussed. Examination of homogenates of the cortex and medulla showed that the latter tissue has a considerably lower oxidative rate than does the cortex. Marked *in vitro* effects of cortisone and desoxycorticosterone on oxidations in adrenal, cardiac muscle and kidney homogenates were observed. The significance of these effects awaits further study.

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RECIPROCAL RELATION BETWEEN THE HYPOPHYSIS AND ADRENALS IN FETAL RATS: EFFECTS OF UNILATERAL ADRENALECTOMY AND OF IMPLANTED CORTISONE, DOCA AND SEX HORMONES[1,2,3]

RALPH L. KITCHELL AND L. J. WELLS

From the School of Veterinary Medicine and the Department of Anatomy of the College of Medical Sciences, University of Minnesota, St. Paul and Minneapolis, Minn.

WE HAVE recently presented some observations which suggested that in the fetal rat the hypophysis cerebri secretes a hormone (ACTH), that this hormone stimulates the secretion of an active principle(s) by the adrenal cortex and that the cortex does not produce any appreciable quantity of androgen, if any (Kitchell and Wells, 1951). It was observed that in fetuses of the 20th day the adrenal cortex has two zones. In those of the 22nd day it has three: outer, intermediate and inner. The cells of the intermediate zone are large, and they contain droplets of osmiphilic lipids. Implanted ACTH speeds up the growth of the cortex, while the removal of the fetal hypophysis by decapitation retards it. This retardation can be prevented by means of injected ACTH. Adrenalectomy does not inhibit the growth of the reproductive organs in the male.

The view that ACTH from the fetal hypophysis stimulates the secretion of an active principle by the developing cortex is supported by the results of subjecting fetuses to unilateral adrenalectomy (Kitchell, 1950a; cf. Tobin's experiments, 1939). The remaining adrenal undergoes a compensatory enlargement. This hypertrophy raised the question of whether there is in the fetus a functional, reciprocal relation between the hypophysis and adrenal cortex.

The main purpose of the present article is to report new observations

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[2] This paper has been designated No. 2691, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[3] The cortisone was supplied by the Merck Laboratories, through the courtesy of Dr. L. Michaud. The desoxycorticosterone acetate (Percorten), testosterone propionate (Perandren), progesterone (Lutocylin) and estradiol benzoate were furnished by Ciba Pharmaceutical Products, Inc., through the generosity of Dr. Ernst Oppenheimer, Dr. Cornelius H. Sullivan and Mr. Fred H. Houghton.

which suggest that the foregoing question should be answered in the affirmative. As we shall see, implanted cortisone prevented that compensatory hypertrophy of the intact adrenal which ordinarily follows unilateral adrenalectomy. The paper also presents, as implied in the title, the results of related experiments. Some of these results have been recorded in abstracts (Kitchell, 1950b, 1951).

MATERIALS AND METHODS

In rats of the Sprague-Dawley strain, 68 experimental fetuses and 61 controls were obtained from 48 pregnancies which were dated from the time of observed copulation. The treatment was begun on the morning of the 20th day (19½ days) and ended just before term.

The methods of treatment have been described (Wells, 1950; Kitchell, 1950a; Kitchell and Wells, 1951). In short, the pellets of hormone to be implanted were prepared in advance, dried in a desiccator and weighed; each pellet weighed, on the average, 1.0 mg. The uterus of an anesthetized pregnant rat was exposed by opening the peritoneal cavity. In Groups D through H (Table 1) a tiny incision was made in the uterus and fetal membranes, a pellet of hormone was implanted under the exposed skin of the fetus and the uterine incision was closed. In Groups A, B and C the fetus was first taken out of the uterus and fetal membranes without injuring its lifeline (umbilical cord) and then subjected to left adrenalectomy. In Groups B and C a pellet of hormone was subcutaneously implanted. Each treated fetus in Groups A, B and C (fetus "extrauterinized" but left attached to the uterus by the umbilical cord), was placed in the body cavity of the mother. After all steps in the treatment had been completed the maternal incisions were sutured, the anesthesia discontinued and the mother taken to her cage.

At 52($\pm$1) hours after the treatment had been finished, each experiment was ended by killing the mother; at this time, about one hour before expected parturition, the determined age of the fetuses was approximately 21 days and 15 hours. The experimental fetus(es) and a litter-mate control which up until now had remained untouched in the uterus were removed, weighed, killed and eviscerated for the purpose of exposing the adrenals; in each experimental fetus in Groups B through H the implanted pellet of hormone was removed and stored in a desiccator in order to obtain at a later time the final, dried weight. The two eviscerated bodies (male and male or female and female) were fixed in Bouin's solution.

After fixation the adrenals were dissected out and removed. The right gland was sectioned in series at 10μ, and the sections were stained with hematoxylin and eosin. In Groups D through H the left adrenal was sectioned at 4μ but not in perfect series. Selected sections were stained by the technique of Masson.

The volume of the right adrenal was determined from the series of stained sections by the paper-weight method of Hammar (1914). Every sixth section was projected at a magnification of 50 diameters and drawn, the tracings were excised and weighed and an adaptation of Boyden's formula (1940) was used.

OBSERVATIONS

This section of the paper should be introduced by calling attention to our earlier observation that the adrenals of male fetuses are morphologically similar to those of female fetuses and that the "adrenal volume/body

TABLE 1. VOLUMETRIC DATA ON THE RIGHT ADRENAL

Fetuses		Treatment	Body weight (gm.), av.	Volume ($mm^3 \cdot 10^2$), av.	Vol. ($mm^3 \cdot 10^2$) / Body wt. (g)		
Group	No.				Av.	P_t[1]	E/C
A	14 E[2]	Unilateral adrenalectomy	4.42	51.7	11.7	.001	1.38
	14 C		4.55	39.1	8.5		
B	6 E	Unil. adr. and cortisone	4.50	31.7	7.0	.008	.87
	6 C		4.54	36.8	8.1		
C	6 E	Unil. adr. and DOCA	4.60	53.0	11.5	.02	1.35
	6 C		4.69	40.2	8.6		
D	7 E	DOCA	3.93	42.1	10.7	.9	1.16
	7 C		4.36	40.2	9.2		
E	9 E	Cortisone	4.10	33.3	8.1	.4	1.08
	9 C		4.64	34.8	7.5		
F	8 E	Androgen	4.74	61.1	13.0	.047	1.13
	8 C		5.13	58.8	11.5		
G	8 E	Progesterone	5.14	67.6	13.1	.5	1.04
	8 C		5.27	67.0	12.6		
H	10 E	Estrogen	4.31	57.9	13.4	.9	1.11
	10 C		4.56	55.2	12.1		

[1] Values of .01 to .05 would suggest that the several "litter differences" (C minus E) are significant; those smaller than .01, highly significant.
[2] E, experimental; C, control.

weight" is essentially the same in the two sexes (Kitchell and Wells, 1951).

Accordingly, in the present study, the averaged data on each group in Table 1 were obtained from males and females. However, in a particular group (aside from the exceptions to be noted below), the experimental males were matched by an equal number of control males; similarly, the experimental females were matched by an equal number of control females. In those exceptional litters in which there were two experimental fetuses of the same sex, only one control fetus (of the same sex) was used; in computing averages, the data on this single control were entered in duplicate; actually there were 13 control fetuses in Group A, 6 an D, 5 in G and 8 in H. There were also three exceptional litters in which, for one reason or another, the experimental fetus and its control were of the opposite sex (one litter in Group B, one in C and one in E).

Before taking up the physiological effects of the various treatments we

should deal briefly with the subject of absorption of hormone from the implanted pellets. In each pellet the determined amount which had been absorbed was obtained by subtracting the final weight from the initial weight. The ponderal differences ranged from 0 to .4 mg. (maximum of .2 mg. in Group B, .2 in C, .2 in D, .4 in E, .3 in F, .4 in G and .4 in H). The "experimental error" in weighing such tiny pellets on an analytical balance must have been a factor in the differences observed. In any event there did not seem to be any close correlation between the amount "absorbed" and the magnitude of the physiological effects. It does not seem necessary to give this subject further attention.

Controls. The adrenals had a capsule, a cortex of three zones (outer, intermediate and inner) and a medulla. The capsule and intermediate zone are best shown in Fig. 3, while the medulla and outer zone are best shown in Fig. 1.

In concentrating upon the three cortical zones, the cells of the outer zone were small and tightly packed. The cytoplasm was scant and non-vacuolated. Mitotic figures were present (not illustrated). In contrast the cells of the intermediate and inner zones were larger, the cytoplasm more abundant and the mitotic figures fewer. The principal difference between the intermediate and inner zones, aside from topographical position, was the degree of cytoplasmic vacuolation. The vacuoles were most numerous in the intermediate zone.

Effects of unilateral adrenalectomy (Group A). This group is composed of fetuses from seven new litters and from the litters which were presented in an earlier paper (Kitchell, 1950a). The effects were the same as those already reported.

The observations in this group constitute the standard for judging the effects in Groups B and C. Figures 1 and 2 are reproduced at such low magnification that they should be supplemented by a short written account.

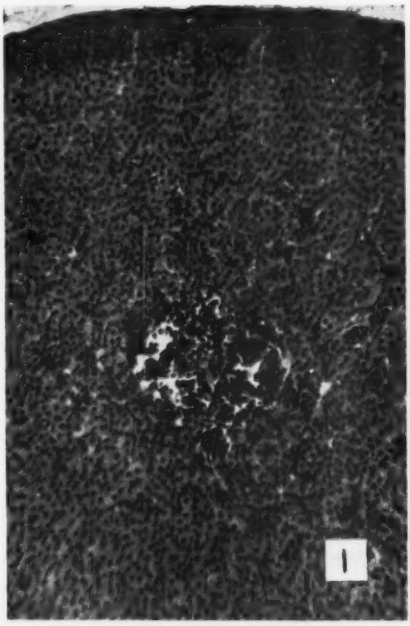
In each litter the sectioned right adrenal of the experimental fetus (E)

FIG. 1. Section of the right adrenal of a control fetus. Between the thin capsule (top) and the medulla (center) is the cortex. At the magnification presented the width of the outer zone of the cortex is less than 1 cm., and the boundary between the intermediate and inner zones cannot be seen. Group A, Litter 1044, 10μ, hematoxylin and eosin, $\times$ 158.

FIG. 2. Compensatory enlargement of the right adrenal after left adrenalectomy. Group A, Litter 1044, 10μ, hematoxylin and eosin, $\times$ 158.

FIG. 3. Left adrenal of a control fetus. This photograph illustrates the capsule and two of the cortical zones, outer and intermediate, but does not include the inner zone. In the intermediate zone observe the sinusoids, large cells and cytoplasmic vacuoles. Group F, Litter 788, 4μ, technique of Masson, $\times$ 620.

FIG. 4. Left adrenal of an experimental fetus in which a pellet of androgen had been implanted under the skin. Group F, Litter 788, 4μ, technique of Masson, $\times$ 620.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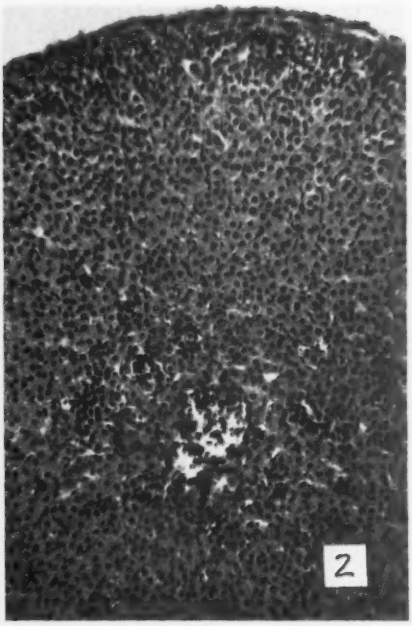
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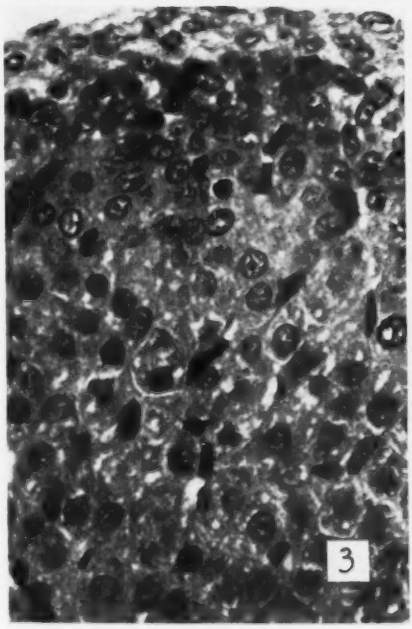
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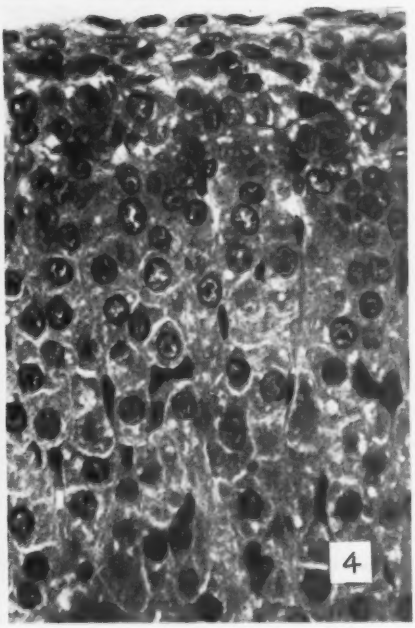
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and that of the control fetus (C) were simultaneously examined with the aid of a microcomparator. It was found that the cells of the outer cortical zone were largest in E. This difference involved both cytoplasm and nucleus. The intermediate zone was widest in E, and its cells were largest in E. The thickness of the inner zone was about the same in E and C. The cells of the inner zone were largest in E, and the cytoplasmic vacuoles were most numerous in E.

In Table 1 the average E/C value of 1.38 and the P_t value of .001 reflect the fact that in 13 of the 14 cases the "litter E/C value" was greater than unity (the 14 values ranged from .90 to 1.79). All the observations in Group A make it clear that in a large majority of the experimental fetuses the right adrenal had undergone a compensatory hypertrophy.

Effects of unilateral adrenalectomy and implanted cortisone (Group B). The stained sections were microscopically examined with the aid of a microcomparator. It was found that morphologically the E and C adrenals were essentially alike.

The quantitative data indicate that the E adrenals were actually the smallest and that the E and C body weights were almost identical (cf. the average E/C value of .87, Table 1). The six "litter E/C values" were all smaller than 1.00, the range being .79 to .96. The quantitative differences seem to be statistically significant.

Two of the litters are of special interest. Each had two unilaterally-adrenalectomized fetuses. One of these fetuses was an "experimental member" of Group B (cortisone implanted), and the other was an "experimental member" of Group A (cortisone not implanted). The two "litter E/C values" for Group B were .85 and .96 (cortisone implanted), while the two for Group A were, respectively, 1.36 and 1.50 (cortisone not implanted).

Effects of unilateral adrenalectomy and implanted desoxycorticosterone acetate (Group C). The effects were like those in Group A (unilateral adrenalectomy alone). The quantitative data showed that in each of the six litters the E adrenal was larger than the C adrenal and that the E and C body weights were almost equal. The six "litter E/C values" were all greater than 1.00 (range, 1.07 to 1.80). The quantitative differences would seem to be significant (P_t of .02).

One litter deserves special attention. It had two unilaterally-adrenalectomized fetuses. One was an "experimental member" of Group C (DOCA implanted), while the other was an "experimental member" of Group A (DOCA not implanted). The "litter E/C values" were, respectively, 1.80 and 1.79.

Effects of DOCA and cortisone (Groups D and E). There did not seem to be any significant effects upon the adrenals and body weight. A micro-

scopical examination of the sectioned adrenals in the field of a microcomparator showed that the minute anatomy of the E gland was similar to that of the C gland.

Effects of testosterone propionate (Group F). The implanted androgen had not caused any marked effects upon the adrenal. Figures 4 and 3 show that in one litter the minute structure of the E gland was similar to that of the C gland.

Judging from the quantitative data, it is problematical as to whether the androgen had slightly stimulated the growth of the adrenal. The P_t value of .047 is barely below the upper limit of a commonly-accepted range which suggests statistical significance (range from .01 to .05, Treloar, 1942).[4]

Effects of progesterone and estradiol benzoate (Groups G and H). The E and C adrenals were histologically and cytologically similar. Volumetrically they were somewhat different, but the difference is not statistically significant.

DISCUSSION

We have recorded one line of experimental evidence of a functional, reciprocal relation between the fetal hypophysis and adrenals, namely the observation that in unilaterally-adrenalectomized fetuses a compensatory hypertrophy of the intact adrenal may be prevented by means of implanted cortisone. Although our view of the existence of a reciprocal relation has not yet achieved the status of a thesis, it would seem to be a useful working hypothesis.

This hypothesis is supported by our observations which were cited in the introduction, by the results of other work in fetuses and by a study in chick embryos. It has been reported that: a) "hypophysectomy"[5] retards the growth of the adrenals in fetal rats (Wells, 1947, 1948; Domm and Leroy, 1951), mice (Raynaud and Frilley, 1950) and rabbits (Jost, 1948), decreases the number of liposomes in the adrenal cortex (intermediate zone) of fetal rats (Wells, 1949) and alters the amount of ascorbic acid in the adrenals of chick embryos (Case, 1951), b) this retarded growth of the adrenal and reduction of its liposomes may be prevented by means of injected ACTH (Wells, 1948, 1949), c) an increase in the weight of the adrenals of fetal rats may be produced by adrenalectomizing the pregnant

[4] In a preliminary study of the data on Group F, an error in making the t-test gave a P_t value of .03; this led to what may have been an unwarranted conclusion that androgen had "slightly increased the volume of the adrenal" (Kitchell, 1950b).

[5] This "hypophysectomy" was accomplished by irradiation (mice) or by decapitation (rats, rabbits, chicks); in chick embryos, certain workers failed to observe a retarded growth of the adrenals after hypophysectomy by irradiation (Wolff and Stoll, 1937; Stoll, 1939) or by decapitation (Fugo, 1940).

mother (Ingle and Fisher, 1938; Walaas and Walaas, 1944; Houssay, 1945)[6] and d) this increase may be prevented by introducing DOCA into the maternal circulation (Walaas and Walaas, 1944; Houssay, 1945).

Our working hypothesis is also supported by certain observations in infants and newborn rats. It has been reported that the adrenals of premature infants are able to respond to "stress" in the same manner as those of the adult (Venning, Randall and Gyorgy, 1949). These workers also found that in premature infants the quantity of excreted glucocorticoids is about the same as that in full-term infants. In newborn rats, van Dorp and Deane (1950) have presented some histological and cytological evidence of the secretory activity of the adrenal cortex.

The hypothesis is similar to one advanced by MacKay and MacKay (1938) in order to explain their observation that injected extracts of the adrenal cortex prevented a compensatory hypertrophy of the adrenal in rats which had been unilaterally-adrenalectomized during the postnatal period. This view of the existence of a reciprocal relation between the hypophysis and adrenals during the postnatal period is supported by numerous observations (Sayers and Sayers, 1949) including those on the effects of injected Compound E (Lewis, Rosenberg and Wilkins, 1950). Lewis, et al., reported a decrease in the size of the adrenals in injected but non-hypophysectomized rats and an absence of it in injected animals which had been hypophysectomized and then maintained "on a constant dose of ACTH." They concluded that the "adrenal atrophy produced by treatment with Cpd. E is caused chiefly if not entirely by inhibition of the production of ACTH".

The question arises as to why in our experiments the implanted cortisone had failed to reduce the size of the adrenals (Group E). Although the answer is not apparent, it should be pointed out that the period of treatment was, of necessity, relatively short.[7] It may be added that the absorption of some of the implanted hormone would seem to have occurred since implanted cortisone prevented any compensatory hypertrophy in Group B.

In Group B there may have been an actual reduction in the size of the adrenals, as suggested by the volumetric data alone (P_t of .008). However, the volumetric difference between the E and C adrenals was not associated with any detectable changes in the minute structure of the adrenal cortex.

While it is not clear as to why the implanted DOCA and sex hormones had failed to produce any appreciable effects, this failure is explained in

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[6] McKeown and Spurrell (1940) carried out similar experiments, and did not find any perceptible difference between the experimental adrenals and control glands.

[7] In fetuses deprived of the hypophysis by decapitation, for example, the greatest effects upon the adrenal were noted in those cases in which the experimental period had been the longest, four days (Wells, 1948; Kitchell and Wells, 1951).

part by the observations of other workers. Sayers and Sayers (1949) found that DOCA and progesterone are less effective than cortisone in inhibiting the secretion of ACTH by the hypophysis of the rat after birth, the ratios being, respectively, 1 to 4 and 1/50 to 4. Whereas Walaas and Walaas (1944) reported the prevention of an enlargment of the fetal adrenals by introducing DOCA into the circulation of the adrenalectomized mother, the length of the period of treatment was doubtless an important factor; they began the treatment on the 16th day of pregnancy and continued it up to term. Parmer, Katonah and Angrist (1951) observed that introduced DOCA did not influence the ponderal growth of rats during the neonatal period.

In our work the data suggest that the implanted hormones had not caused any perceptible modification of the growth of the fetuses. Other investigators have reported that large doses of cortisone inhibit the growth of immature rats (Wells and Kendall, 1940; Parmer, Katonah and Angrist, 1951). However, Leroy and Domm (1951) did not note any effects upon the weight of fetuses which had been subjected to cortisone.

Returning to our principle hypothesis, we have considered the question of whether the enlargement of the adrenals of unilaterally-adrenalectomized fetuses could be due in part to the action of ACTH from the maternal hypophysis or from some other source (placenta?). We would answer this question in the negative since the removal of the fetal hypophysis by decapitation retards the growth of the adrenals and since we are not aware of evidence that ACTH can cross the placental barrier (see Houssay's study of the adrenals in fetuses from mothers which had been adrenalectomized, 1945). In regard to the placenta Greer (1949) studied the adrenals of hypophysectomized pregnant rats, and concluded that the placenta does not produce ACTH; while his observations also suggest that ACTH of fetal origin had not reached the maternal circulation, he did not mention this.

Our working hypothesis does not seem to be discredited by certain experiments which failed to demonstrate the secretion of ACTH in immature rats and premature infants. Jailer (1950) used the immature rat, injected epinephrine, killed the animal "between one and two hours later," determined the ascorbic-acid content of the adrenals and noted that the amount of ascorbic acid had not been reduced in any case in which the rat was younger than 8 days; lowered temperature did not reduce the amount in rats younger than 16 days. Jailer, Wong and Engle (1951) used premature infants, subjected each to one injection of epinephrine, counted the eosinophils in three samples of blood (one sample drawn before the injection, the others at two hours and four hours after the injection) and observed that the number of eosinophils had not been significantly decreased in any case in which the infant was under nine days of age. That these techniques

may have certain shortcomings when they are used in newborn rats and premature infants is suggested by the statment that "the infant rat will . . . respond to a sustained stress over a period of five days" (Jailer, Wong and Engle, 1951, p. 191).

Finally the hypothesis should be considered in the light of the fact that pregnancy prolongs the life of adrenalectomized dogs (Rogoff and Stewart, 1927) and tends to relieve temporarily the symptoms of Addison's disease (Thorn, 1949). While it has not been shown that the adrenals are involved in these phenomena, we do not know of any observations which would exclude them as a contributing factor. The action of progesterone seems to be one factor since injections of this hormone will prolong the life of adrenalectomized ferrets (Gaunt and Hays, 1938) and rats (Schwabe and Emery, 1939; Greene, Wells and Ivy, 1939). A study of the quantity of urinary ketosteroids in a pregnant woman with Addison's disease attracts attention (Samuels, Evans and McKelvey, 1943). The quantity remained very low until the 6th month, then rose to a level of 11 to 15 mg. per day and, after partuition, immediately dropped to the original low; the Addisonian symptoms fully returned after the baby had been born. Samuels, Evans and McKelvey presented the suggestion that the adrenals of the fetus might have been the source of these ketosteroids.

SUMMARY AND CONCLUSIONS

In fetuses which had been subjected to left adrenalectomy on the morning of the 20th day and killed just before term, stained serial sections of the right adrenal were first studied microscopically with the aid of a micro-comparator and then used in determining the volume of the gland (paper-weight method). It was found that the intact adrenal had become enlarged. In similar fetuses in which a pellet of cortisone (1.0 mg.) was implanted under the skin, this compensatory hypertrophy of the adrenal was prevented. Implanted desoxycorticosterone acetate (DOCA) did not prevent the compensatory hypertrophy.

Fetuses with both adrenals intact were subjected to the subcutaneous implantation of a pellet of hormone (cortisone, DOCA, testosterone propionate, progesterone or estradiol benzoate). The treatment did not produce any appreciable change in the volume of the adrenal nor in the minute anatomy of the cortex.

The prevention of an enlargment of the adrenal in a series of unilaterally-adrenalectomized fetuses by means of implanted cortisone constitutes one line of evidence that in fetal rats there is a functional, reciprocal relation between the hypophysis and adrenals. Our view of the existance of this reciprocal relation would seem to be a useful working hypothesis.

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A SIMPLE METHOD FOR THE MEASUREMENT OF THE OXYGEN CONSUMPTION AND HEART RATE OF RATS

B. GRAD

Departments of Psychiatry and of Anatomy, McGill University, Montreal

WHILE engaged in a study of the effect of thyroxine on the oxygen consumption and heart rate, the need arose for a simple means of measuring the oxygen consumption and heart rate of a large number of rats daily. Because existing techniques were found to be unsuitable for such studies, a simple apparatus was devised in which the only special piece of equipment required was an electrocardiogram for measuring the heart rate. This paper will describe this method and, as an example of the type of use to which it can be put, the response of the oxygen consumption and heart rate of rats to graded doses of thyroxine will be reported.

METHODS AND TECHNIQUES

Oxygen Consumption: The animal was placed in a 48 oz. jar, A, (Fig. 1) which contained soda-lime to absorb CO_2 and water. Closing the jar was a rubber stopper fitted with a T-tube, B, one arm of which led to a manometer, C, and the other to a 300 cc. florence flask, D, containing oxygen at atmospheric pressure. The flask, D, had been prepared with a test-tube, KG, connected by a siphon, I, to the water bath, J, in which the apparatus was immersed. The siphon and test-tube were filled with water at the beginning of the determination.[1] The manometer, C, indicated a subatmospheric pressure in A as the animal in A consumed oxygen, causing water to flow into the flask. D, via the siphon, I, and the test-tube, KG. After 15 minutes the jar, A, was disconnected from the flask, D, at the joint, O. By applying pressure at O, the water in the flask, D, was blown through the tube, FP, into a graduated cylinder and measured. This volume of water corresponded to the volume of oxygen consumed to the nearest cc. Because the oxygen consumption of a mammal is a function of the $\frac{3}{4}$ power of its body weight, the results were expressed as cc. of oxygen consumed per unit of the $\frac{3}{4}$ power of body weight in grams (Kleiber, 1947).

In practice six, and more recently, twelve such units were used simultaneously, enabling one person to carry out 40 to 50 determinations in an afternoon. Rats were trained to be quiet during the determination by placing them in the jars for an hour daily for several days prior to the measurements. Repeated temperature measurements of the rat chamber revealed that it did not rise more than 1° C. while the oxygen consumption was being measured.

Heart Rate: The heart rate was also measured with the rat sitting in the same soda-lime-containing jar, A, as above, the jar being covered with wire gauze and not with a rubber stopper as in Fig. 1. Two safety pins, one fastened in the skin on each side of the

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[1] Dr. E. Robillard of the Department of Physiology of the University of Montreal suggested the use of the test-tube as described above.

animal, were used as electrodes. The pins were connected by thin wire leads to terminals on a control board. When the animal became quiet, two leads from an electrocardiograph were plugged into these terminals and a recording was taken on time paper. The results were expressed as beats per minute.

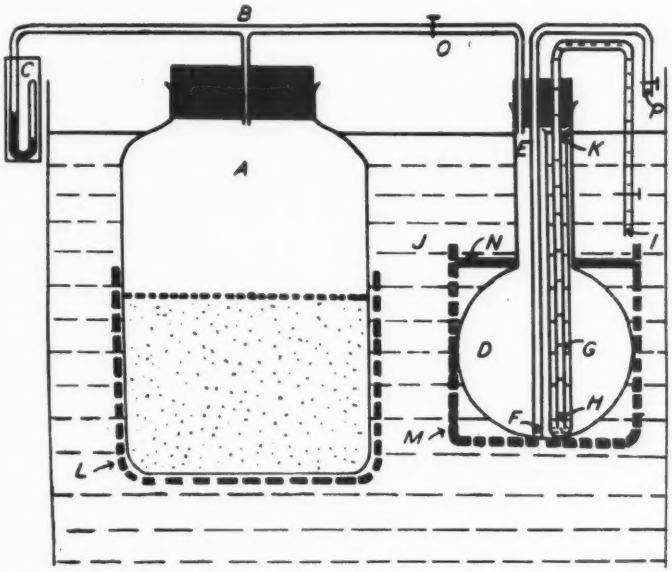

FIG. 1. Apparatus for measuring oxygen consumption in rats and mice. L, M are perforated metal baskets supporting the entire apparatus in the water bath (J), which is kept at 28±1° C. during measurements. These metal baskets are unnecessary if the water in the bath is not too deep. Anchors (N) keep the florence flask in the bath. The whole apparatus is brightly illuminated to render the rat, a nocturnal animal, less active.

In practice the control board had 50 pairs of terminals, each pair being numbered and connected to a rat in a jar with the corresponding number.

To measure oxygen consumption and heart rate of a rat simultaneously, the two leads of the electrocardiograph were plugged into two terminals which passed through the rubber stopper of the jar used for the measurement of the oxygen consumption.

The determinations were carried out in the same chamber in which the animals were housed, a chamber in which the temperature was maintained at 29±2°C., and in which the pressure varied from 760 to 780 mm. Hg. Because of this constancy of conditions in the experimental chamber, no corrections were made in the oxygen consumption results for temperature and pressure changes in the room. This chamber was also automatically illuminated from 8:00 A.M. to 8:00 P.M. Wistar rats, fed on purina fox chow and water ad lib. were used and were not fasted prior to the determinations. All experiments were carried out on unanaesthetized animals, and measurements were made at the same time of day.

Statistical analyses were carried out to determine the significance of differences (Snedecor, 1948). Probability values less than 0.05 were considered significant.

RESULTS

To test the reliability of the above apparatus, the oxygen consumption of six male albino rats, weighing between 103 and 116 grams, was measured repeatedly on the same afternoon for 60, 20, 10 and 80 minutes respectively (Table 1). The duration of each measurement was changed on the assumption that the longer the time taken to measure the oxygen consumption the greater were the chances for weaknesses in the apparatus to reveal themselves (leaks, deficient absorption of the CO_2 by soda-lime, etc.).

TABLE 1. VARIATIONS IN THE OXYGEN CONSUMPTION AND HEART RATE OF MALE RATS

	Oxygen consumption			Heart rate	
Rat	Mean ± standard error (cc./hr./gram$^{3/4}$)	Coefficient of variation	Rat	Mean ± standard error (beats/minute)	Coefficient of variation
A*	5.8±0.1	2.4	G*	366±17	10
B	5.6±0.2	6.6	H	398±13	7
C	5.1±0.4	16.4	I	355± 6	4
D	5.5±0.2	6.1	J	342± 8	5
E	5.5±0.2	8.1	K	350± 4	2
F	5.4±0.1	1.8			
A to F (inclusive)	5.5±0.1	8.2	G to K (inclusive)	362± 6	8
Time Interval (minutes)			Time Taken		
10†	5.5±0.2	9.2	9 A.M.‡	373±17	10
20	5.6±0.2	7.1	11 A.M.	370± 6	4
60	5.5±0.2	10.7	2 P.M.	354± 7	4
80	5.4±0.2	6.8	4 P.M.	351±19	12

* Four readings of the oxygen consumption and heart rate were taken per rat.
† The oxygen consumption of the above six rats was measured at each of the four time intervals.
‡ The heart rates of the above five rats were measured at each of the four times of the day.

A test of the reproducibility of the heart rate technique was also made on five male albino rats, weighing between 160 and 195 grams, whose heart rate was measured four times between 9:00 A.M. and 4:00 P.M. (Table 1).

The mean oxygen consumption was 5.5 cc./hr./gram$^{3/4}$ with a coefficient of variation of 8.2, and the mean heart rate was 362 beats per minute with a coefficient of variation of 8. There was no significant difference between the oxygen consumption results in which the duration of measurement varied from 10 to 80 minutes; the heart rate could also be measured at any time between 9:00 A.M. and 4:00 P.M. without significant variation.

This apparatus was also applicable for the measurement of oxygen consumption and heart rate in mice (Grad, 1949).

Because of its intended use in studies of the biological activity of thyroxine, the effect of graded doses of racemic sodium thyroxine on the oxygen consumption and heart rate of albino rats was investigated in eight thyroidectomized adult male rats weighing between 160 and 220 grams. The measurements were begun five weeks after thyroidectomy, at which time the animals were divided into two groups of four. A single subcutaneous injection of 100 μg. of thyroxine was then administered to the rats of Group 1, the rats of Group 2 receiving the same volume of distilled water.

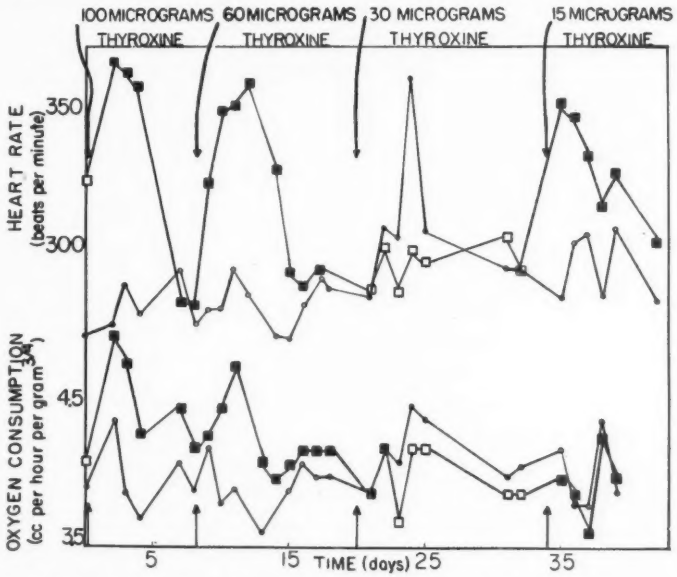

FIG. 2. Influence of various doses of subcutaneously administered thyroxine on the oxygen consumption and heart rate of thyroidectomized rats. Each square represents the results of Group 1, and each circle those of Group 2, four rats being in each group. Closed symbols represent thyroxine-treated rats, and open symbols represent controls. Statistical analysis of the results of Group 1 vs. Group 2 is as follows:

Oxygen Consumption: Days 3, 4, 7: $P < 0.001$; Days 10, 11, 13: $P < 0.001$; Day 23: $0.3 > P > 0.2$. Days 37, 38: $0.4 > P > 0.3$; Heart Rate: Days 2, 3, 4: $P < 0.001$; Days 9, 10, 11, 12, 14: $P < 0.001$; Day 24: $0.01 > P > 0.001$; Days 35, 36: $P < 0.001$.

When the stimulating effects of the 100 μg. dose wore off, the procedure was repeated with a 60 μg. dose, and similarly with a 30 and 15 μg. dose. The 30 μg. dose was administered to the rats of Group 2 while those of Group 1 served as controls.

Significant increases in heart rate were observed at all dose levels tested, but significant increases in the oxygen consumption were obtained only with 100 and 60 μg. of hormone (Fig. 2).

DISCUSSION

A mean oxygen consumption value of 5.5 cc./hr./gram$^{3/4}$ obtained in control tests (Table 1) was in agreement with the value of 6.1 cc./hr./gram$^{3/4}$ calculated from the data of Kibler and Brody (1942), the only comparable study carried out on unfasted male rats of the same weight. The control mean heart rate value of 362 beats per minute (Table 1) fell in between the reported values, the lowest reported being 264 (Moses, 1946) and the highest 431 beats per minute (Leblond and Hoff, 1944). This agreement with reported values together with the reasonably small coefficients of variation indicated that this apparatus was suitable for the measurement of the oxygen consumption and heart rates in rats. In addition, this apparatus has proved to be especially valuable in several investigations in which a large number of rats had to be measured daily for several weeks (Leblond and Grad, 1948; Grad, 1949; Grad and Leblond, 1950).

Several points of technique should be emphasized: the animals were not fasted prior to the measurements because in many of the studies they had received such strenuous treatment as massive thyroxine injections combined with partial hepatectomy, etc. It was felt that the additional stress of an over-night fast, a procedure which can produce adrenal hypertrophy and intestinal hemorrhage in thyroxine treated rats (Leblond, personal communication), would have raised the mortality to the point where the studies could not be completed. Moreover, measurements made on normal unfasted rats were clearly reproducible, as indicated by the low coefficients of variation (Table 1). Part of the reason for this low variability was that the oxygen intake was always measured between 1:00 P.M. and 5:00 P.M., at which time the rats' stomachs were regularly most empty (Dougherty, Gross and Leblond, 1951). The rats normally ate during the night, at which time the oxygen consumption measurements were also the highest (Grad, 1949).

Furthermore, the measurements were carried out in a controlled environmental temperature, and only in rats of known age and sex, inasmuch as these factors were also shown to influence oxygen consumption and heart rate (Grad, 1949).

The oxygen consumption response gradually increased as the dose of thyroxine was increased from 15 to 100 μg., indicating a possible use of this apparatus in thyroxine bio-assay (Fig. 2). Although the graded response was steeper for the oxygen consumption than for the heart rate, the latter's response to 15 μg. of thyroxine was greater than the former's, indicating that the heart was more sensitive at this dose level than were many of the other tissues (Fig. 2). This was also borne out by recent studies in which the in vitro oxygen consumption response of heart muscle to a large single injection of thyroxine in thyroidectomized rats was greater

than that of skeletal muscle, diaphragm, liver and kidney (Barker and Klitgaard, 1951).

SUMMARY

A reliable, simple and efficient close-circuit method for measuring the oxygen consumption (Fig. 1) and an electrocardiographic method for measuring the heart rate of rats were described. In this technique the oxygen consumption and heart rate could be measured separately or simultaneously. The reliability of the method was indicated by the agreement found between the results of control tests (Table 1) and the values reported in the literature, the reproducibility by the reasonably small coefficients of variation (Table 1), and the efficiency by its successful use in several studies in which large numbers of measurements had to be made daily over several weeks at a time (Leblond and Grad, 1948; Grad and Leblond, 1950).

Single injections of subcutaneously administered thyroxine in graded dosage from 15 to 100 μg. produced a graded response in the oxygen intake as measured in this apparatus, indicating its possible use in thyroxine bio-assay. Under these conditions, the gradation in the heart rate response was less steep, but at the 15 μg. level was more sensitive than the response of the oxygen intake (Fig. 2).

Acknowledgements

(I am indebted to Dr. C. P. Leblond for his many valuable suggestions and criticisms of the work reported in this paper. This work was supported by a Province of Quebec Mental Health Grant to Dr. Karl Stern of the Gerontologic Unit at the Allan Memorial Institute, and from the National Cancer Institute of Canada to Dr. C. P. Leblond.)

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DIABETOGENIC EFFECT OF GROWTH HORMONE IN THE INTACT FORCE-FED ADRENOCORTICOTROPHIN TREATED RAT*

FRANK L. ENGEL.† ALBERTO VIAU, WILLIAM COGGINS‡ AND WILLIAM S. LYNN, JR.

THE diabetogenic effect of both crude anterior pituitary extract and purified growth hormone in the dog and cat is now well established. Originally demonstrated with crude anterior pituitary extract, (Johns *et. al.*, 1927, Evans, *et al.* 1932, Houssay, 1936, Young 1937 and Lukens and Dohan 1942) both temporary and permanent diabetes have now also been induced in dogs and cats with more highly purified preparations of growth hormone (Cotes, Reid and Young, 1949; Houssay and Anderson 1949; Campbell *et al.*, 1950a, 1950b). As has been emphasized by Young (1944), diabetes is fairly readily precipitated in adult animals wereas in immature ones enhanced growth is the usual response to the same pituitary preparations. In the cat, the diabetogenic effects of pituitary extracts are not apparent following adrenalectomy, but may be restored if the animals are maintained on a constant dose of adrenal extract which itself does not induce glycosuria (Long and Lukens 1936). Purified growth hormone however, is diabetogenic in the totally hypophysectomized dog (DeBodo, Kurtz, Ancowitz and Kiang, 1950). Remarkably small amounts of hormone were found to be effective in the latter animals.

In contrast to the ready production of diabetes in the above species, has been the remarkable resistance of the rat to the diabetogenic effects of anterior pituitary extracts or of growth hormone, even though the rat is quite sensitive to the other metabolic effects of growth hormone. Beyond a mild impairment in glucose tolerance noted by Milman and Russell (1950), there are no reports of growth hormone induced hyperglycemia or glycosuria in the rat with an intact pancreas. In the partially depancreatized rat, however, both temporary and permanent diabetes have followed treatment with relatively large amounts of APE (Long, Katzin and

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‡ F. M. Hanes Student Fellow.

Fry, 1940) and the glycosuria of alloxan diabetic rats has been increased by growth hormone treatment. (Russell 1951). Doses in excess of 3 mg. per 100 gms. per day were necessary to produce this effect in the latter experiments. This may be compared with the dose of 0.1 mg. per 100 gms. found to be effective in the hypophysectomized dog by DeBodo *et al.*, (1950). As in the cat, Long, Katzin and Fry (1940) found that APE was not diabetogenic in the partially depancreatized-adrenalectomized rat, but that adrenal extract would restore the diabetogenic effects of APE.

The weak diabetogenic effect of growth hormone in the rat in turn contrasts with the relatively potent effects of adrenal steroids (Ingle *et. al.*, 1945) and ACTH (Ingle, Winter, Li and Evans, 1945) in this species. Bennett and co-workers (1947, 1948a) have used ACTH and growth hormone under comparable conditions in alloxan diabetic rats and found consistent increases in glycosuria with the former but not with the latter.

The explanation most commonly invoked to account for the different response of the rat and of the dog and cat to growth hormone is that the pancreas of the latter is much less able to sustain a level of insulin secretion adequate to counteract the diabetogenic effect of growth hormone, the islets eventually failing and undergoing degenerative changes. The facts that permanent diabetes has been induced in the cat by massive glucose infusion (Dohan and Lukens, 1948) and prevented by insulin or phlorhizin in the APE treated cat (Lukens *et al.*, 1942, 1943) while permanent diabetes has not been produced in the rat by any means short of pancreatectomy or alloxan treatment lend credence to such an interpretation. The ability of the islet cells of the pancreas of the rat to undergo hyperplasia in response to hyperglycemia is well established (Lukens, 1950).

However, there are reasons for believing that this factor is not sufficient unto itself to account for the differences between the rat and the dog and cat. The most cogent of these is the large amount of APE or growth hormone required to enhance the diabetes of the partially depancreatized or alloxanized rat (Long, Katzin and Fry 1940, Russell, 1951) compared to that for the intact or hypophysectomized dog or cat. In light of the apparent greater sensitivity of the rat than the dog or cat to the diabetogenic effects of adrenal steroids and the well established necessity for adrenal hormone for the diabetogenic response to APE or growth hormone, the possibility suggests itself that a higher level of adrenal activity may be required in the rat to bring out the diabetogenic effect of growth hormone. The relative resistance of both the puppy and the rat, which continues to grow all its life, to the diabetogenic effect of growth hormone, may reflect a relative predominance of growth hormone activity over adrenal activity while the reverse may be true in the adult dog and cat. As suggested by Young (1951) diabetes may result from growth hormone treatment only

when the full growth response to the hormone cannot be achieved, a condition which is full-filled when the organism is subjected to overdosage with ACTH or certain adrenal steroids.

Support for this concept is forthcoming in the following study in which it was found that force-fed rats regularly developed hyperglycemia when treated concurrently with ACTH and growth hormone, but not when treated with either one alone.

METHODS AND MATERIALS

Adult male and female rats of the Vanderbilt strain, weighing 200–300 grams at the start of the experiment were used. Some of the female rats had previously been used for breeding purposes and were used with the thought that they might prove more susceptible to diabetes. The animals were kept in metabolism cages in an air conditioned room at 28–29° C. Urine was collected with thymol as a preservative and analysed for glucose by the method of Benedict (1911). The animals were fed by tube a high

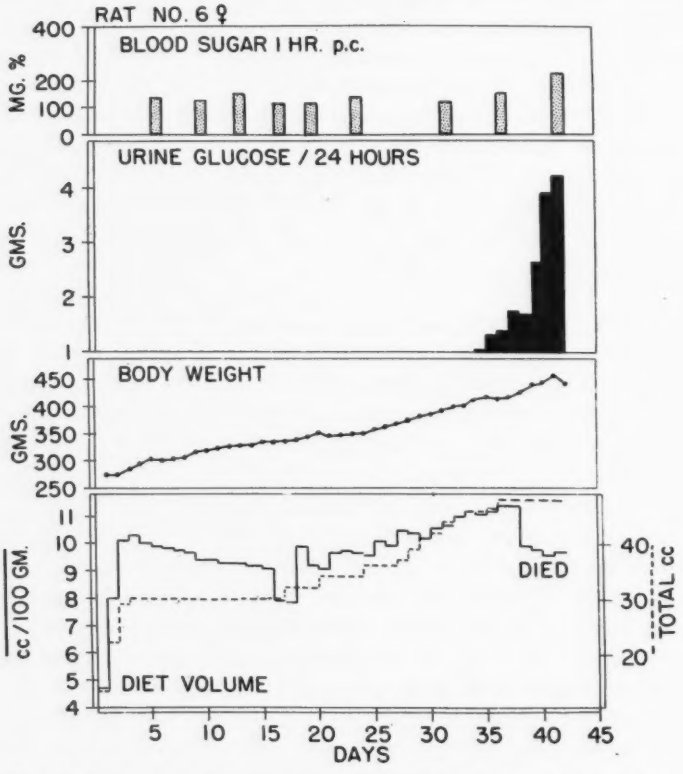

FIG. 1. Production of glycosuria by force-feeding a normal rat with a high carbohydrate liquid diet.

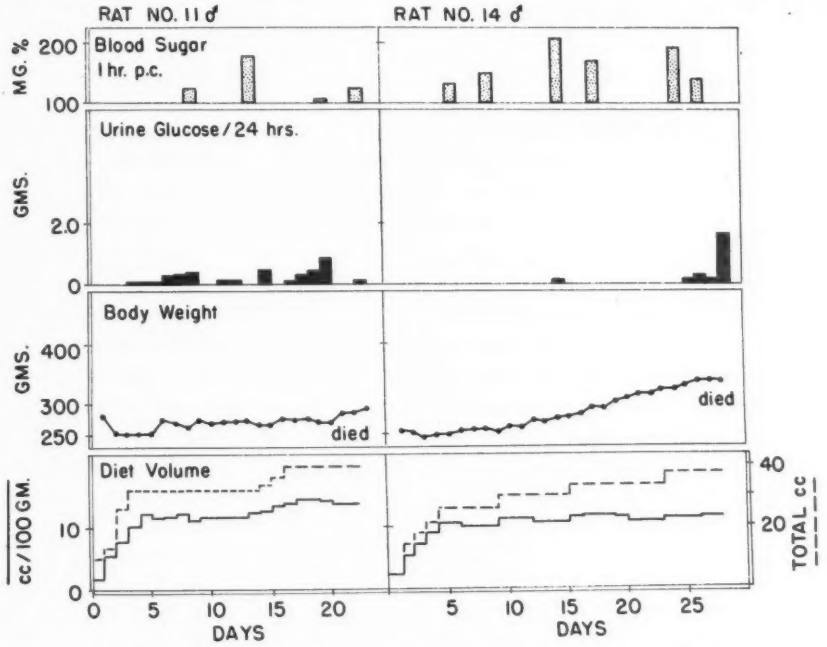

FIG. 2. Influence of variations in diet volumes on the glycosuria of normal force-fed rats.

carbohydrate diet (Ingle, Prestrud, Li and Evans 1947) and were adapted to the diet as suggested by these investigators. This diet contained 0.50 gm. of carbohydrate, 0.13 gm. of protein, 0.005 gm. of fat and 2.57 cal. in each milliliter and was generally administered at 8:30 A.M. and 5:30 P.M. in volumes of 13 to 22 ml. per feeding. With the largest diet volumes, an additional feeding was given at 10 P.M. Blood sugar levels were determined on copper-tungstate filtrates by the method of Somogyi (1945) one hour after the morning tube feeding. The rats were under light nembutal anesthesia when blood was taken from the tail. ACTH and growth hormone were administered subcutaneously in equally divided doses at 8 A.M., 1 P.M., 6 P.M., and 10 P.M., the total doses in each case being indicated on the charts. The hormone preparations used in individual experiments are recorded with the experiments.

RESULTS

Figures 1 and 2 present control data on the effects of variations in total diet and rate of increasing diet on the tolerance of the rats to carbohydrate. In confirmation of the report of Ingle (1946), it was found that glycosuria might be induced in normal rats by either over feeding or too rapid increments of feeding of the high carbohydrate diet. In rat No. 6 (Fig. 1) massive glycosuria began on the 35th day after rapid increases in the diet

brought it to a daily volume of 48 ml. in 3 feedings of 16 ml. each, or a maximum volume of 11.3 ml. per 100 grams. In rats 11 and 14 (Fig. 2) much lesser degrees of glycosuria developed although the diet volumes per 100 grams were greater. Rat No. 11 had a maximal glycosuria of 0.76 grams while receiving 40 ml. of diet or 14.5 ml. per 100 grams whereas rat No. 14 showed no significant glycosuria until the last day when he excreted 1.6 grams on a diet of 38 ml. or 11.2 ml. per 100 grams. The rat died abruptly at this time with no apparent expanation. A fourth rat (No. 15. female, initial weight 271 grams), whose data are not charted, was studied over 45 days while receiving increments of 2 to 4 ml. of diet every 5th day after the 9th day until a total diet volume of 45 ml. per day or 13.9 ml. per 100 gms. was given on the 42nd day. No glycosuria developed until the 45th day when she excreted 1.8 grams of glucose. A fifth rat whose diet was increased 1 ml. a day showed mild glycosuria (0.1 to 0.4 gms. per 24 hours) from the 16th to 21st days while receiving 35 to 40 ml. or 12.1 to 14.1 ml. diet per 100 grams. These results indicate that the total volume of diet and the rate of increasing it must be considered in the interpretation of glycosuria in rats force-fed a high carbohydrate diet.

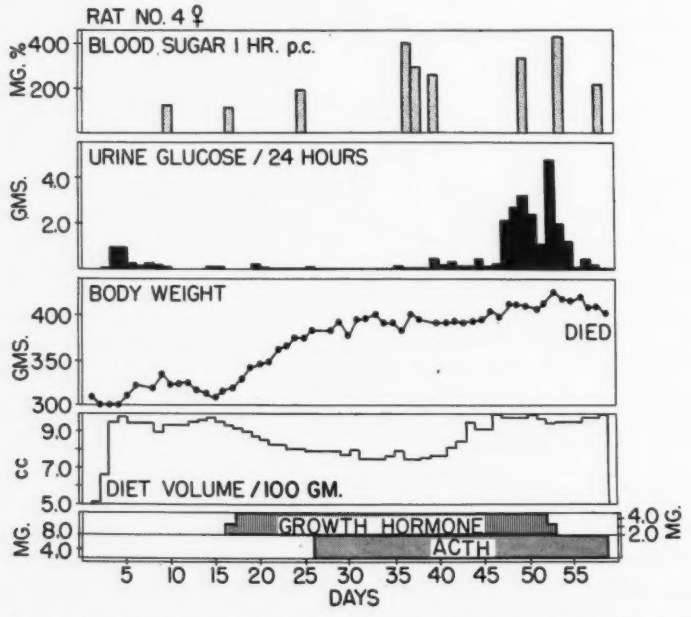

FIG. 3. Effect of combined treatment with growth hormone and ACTH on blood and urine sugars of force-fed rats. Following adaptation to diet, the total volume of diet was 30 ml. per day until the 43rd day. Thereafter, the diet volumes were maintained between 9.0–10.0 ml. per 100 grams per day.

Figures 3, 4, 5, 6, 7 and 8 present data on the effects of ACTH and growth hormone on the carbohydrate tolerance of force-fed rats. Rat number 4, a female, (Fig. 3) weighed about 300 grams at the beginning of the experiment. During adaptation to tube feeding she showed some glycosuria but thereafter while receiving 30 ml. of diet daily had no significant glycosuria during two weeks of observation and two post-cibum blood sugar levels were 122 and 115 mg. per cent. There was an irregular weight gain during this period. Treatment with 5 mg. growth hormone (Armour, Lot 3PKR3) was begun on the 17th day and continued until the 53rd day. There was an immediate sharp increase in the rate of weight gain which was sustained until the 27th day when daily treatment with 8 mg. of ACTH (Armour Lot 79R, dosage expressed in terms of the Armour standard LA-1-A) was begun. The weight curve leveled off while on ACTH and growth hormone and decreased while receiving ACTH alone. On growth hormone alone there was no glycosuria, but a post-cibim bloodsugar

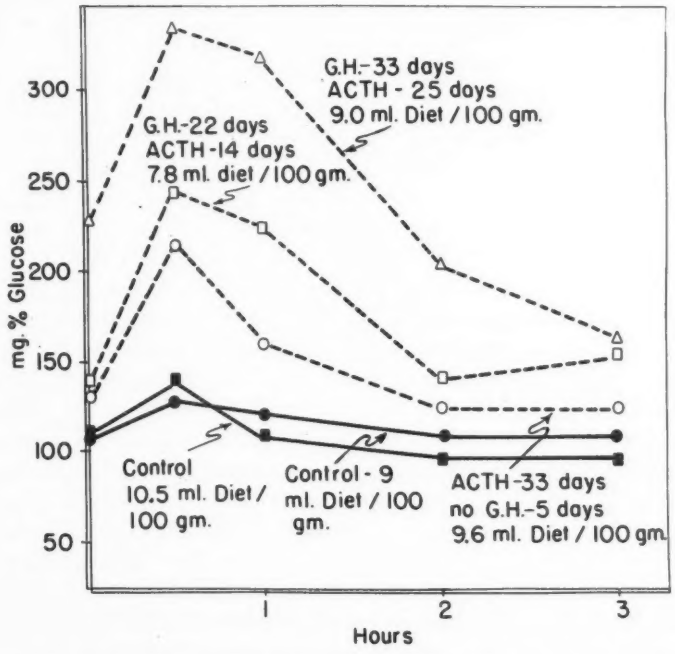

FIG. 4. Glucose tolerance curves in two control rats receiving 9.0 and 10.5 ml. diet per 100 grams compared to those of rat number 4 (Fig. 3). The middle curve from rat number 4 was made when the animal was on a restricted diet and exhibited post-prandial hyperglycemia but no glycosuria, the upper curve when she had glycosuria on ACTH and growth hormone and the lower curve when the animal was receiving ACTH but no growth hormone and was exhibiting no glycosuria.

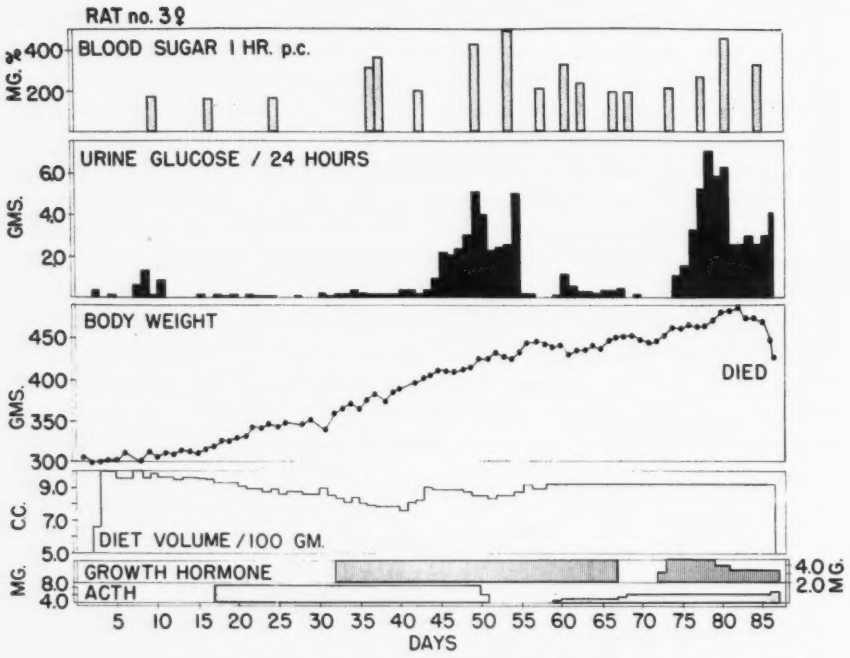

FIG. 5. Effects of combined treatment with ACTH and growth hormone. Until the 43rd day 30 ml. of diet per day were given and from days 43 to 57, 36 ml. of diet administered. Thereafter the diet volume was kept at 9.0 ml. per 100 grams per day.

on day 25 was 190 mg. per cent. On combined ACTH and growth hormone treatment likewise, there was no glycosuria during the first 20 days but post-cibum blood sugar levels of 400, 290 and 244 mg. per cent were recorded. During this whole time, the diet had been kept fixed at 30 ml. per day. In terms of the weight gain of almost 100 grams, this represented a restriction of food intake compared to the control period. The diet volume was therefore increased to correspond to the control period, i.e. 9.5–10 ml. per 100 gms. With this increase in diet a striking glycosuria and hyperglycemia developed which persisted as long as combined treatment was used. On discontinuing growth hormone, glycosuria promptly declined to negligible levels, and progressive weight loss occurred. Regrettably, the rat died on the 60th day as a result of a tube feeding accident.

Glucose tolerance curves on this rat and on controls on a comparable diet are shown in fig. 4. The two lower curves are from control animals. The middle of the 3 upper curves depicts the glucose tolerance of rat No. 4 on day 39 while receiving both ACTH and growth hormone and exhibiting

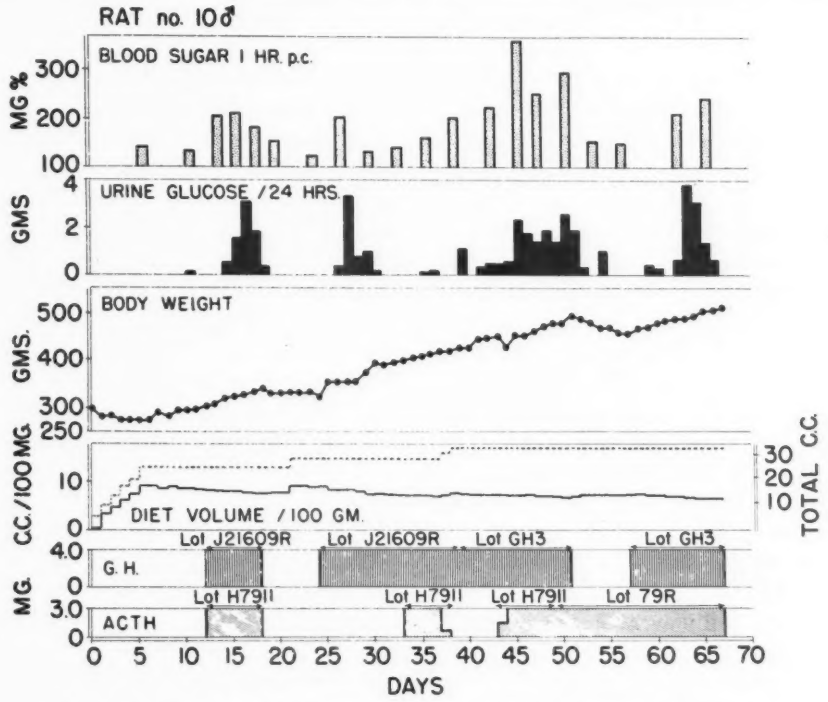

FIG. 6. Effects of combined treatment with ACTH and growth hormone in a normal force-fed rat.

hyperglycemia but no glycosuria. It shows a definitely impaired tolerance, but of a degree, however, which is quite compatible with the absence of glycosuria. The upper curve, done on day 50, when the rat was exhibiting glycosuria, reveals a clearly diabetic curve. The lower curve, showing only slight impairment despite a larger diet, was made on the 58th day while the animal was receiving ACTH but had had no growth hormone for 5 days. These data established quite clearly the presence of diabetes in this rat.

Data on the effects of ACTH and growth hormone on glycosuria which are essentially similar to the above are recorded in fig. 5 (Rat 3). This animal was treated with 8 mg. of ACTH (Lot 79R) beginning on the 17th day and on the 33rd day injections of 5 mg. of growth hormone daily (Lot 3PKR3) were begun. There was no hyperglycemia or glycosuria while receiving ACTH and again hyperglycemia without glycosuria during combined ACTH and growth hormone treatment on a fixed diet of 30 ml. However, marked hyperglycemia and glycosuria resulted when the diet was increased to 36 ml. or 8.0–8.8 ml. per 100 grams daily. When ACTH

was stopped on the 52nd day, glycosuria decreased for 3 days, then increased sharply to 5.0 grams on the 4th day and then abruptly disappeared even though growth hormone treatment was continued. Reinstituting ACTH (Lot H8512) injections at a dose of 1.6 mg. daily caused a slight glycosuria which disappeared when growth hormone was discontinued; treatment with 3.2 mg. of ACTH by itself was not associated with glycosuria but when 5 mg. of growth hormone were again injected marked glycosuria and hyperglycemia ensued. With a fixed dose of ACTH, reduction in growth hormone dosage was associated with corresponding decreases in glycosuria. The rat died on the 87th day after a period of rapid weight loss. Except for the terminal weight loss, this animal gained weight throughout the entire experiment. Weight increments while receiving growth hormone with or without ACTH were slightly greater than when only ACTH was being given. From this experiment, it would appear that there is a minimal level of ACTH necessary to bring out the diabetogenic effect of growth hormone and that, within limits, further glycosuria results from increasing the growth hormone but not the ACTH.

The data on a third doubly treated rat (No. 10) are shown in Fig. 6. The results of this experiment were essentially the same as those in the previous ones, although there were certain differences. This animal gained 200 grams during the experiment, and exhibited glycosuria on the smallest diet of any studied, i.e. 26 to 34 ml. or 6.4 to 8.0 ml. per 100 gms. daily, an intake which is well out of the range which could account for the glycosuria as a result of over feeding. Further more, the dosage of hormones was less than used previously i.e. 3 mg. of ACTH and 4.0 mg. of growth hormone daily. It is also of interest that this rat showed transient glycosuria from the 25th to the 28th days while receiving growth hormone alone (Lot J21609R). According to the manufacturers this lot was free of ACTH. One other animal, rat No. 9, Fig. 7, also developed hyperglycemia and glycosuria while receiving this same lot of growth hormone. However, this latter animal had a rather severe respiratory infection during this period and had greatly enlarged adrenals at autopsy (Table 1). Regrettably, no more of this lot of growth hormone was available so that is was not possible to investigate its diabetogenic properties further. When another lot (GH3) was used in rat No. 10, glycosuria and hyperglycemia resulted only on combined treatment with ACTH. GH3, according to the manufacturers, assayed only 20 per cent of the potency of the Armour growth hormone standard. Hence 20 mg. of this preparation were injected in order to supply 4 mg. of growth hormone. This animal also gained weight more rapidly when receiving growth hormone, with the most rapid increments occurring when growth hormone alone was given and an actual loss of weight when ACTH alone was injected.

TABLE 1

Rat No.	Treatment	Body weight gms.		Organ weights gms. per 100 gm. B. W.						
		Initial	Final	Adrenals	Liver	Pancreas	Heart	Kidneys	Spleen	Comments
6	Diet 42 days	275	447	0.0135	—	—	—	—	—	
14	Diet 29 days	255	339	0.0165	3.91	0.518	—	0.545	0.265	
15	Diet 45 days	271	342	0.0264	6.50	—	0.73	0.660	0.075	
4	Diet 60 days GH 34 days ACTH 33 days	310	400	0.0457	—	—	—	—	—	
3	Diet 87 days GH 50 days ACTH 62 days	300	445	0.0280	6.00	0.590	0.63	0.755	0.400	
10	Diet 76 days GH 46 days ACTH 38 days	300	488	0.0195	5.90	0.512	0.43	0.595	0.143	
2	Diet 89 days GH 39 days ACTH 44 days	293	450	0.0416	7.32	—	—	0.398	—	
9	Diet 27 days GH 13 days	252	319	0.0319	6.90	0.690	—	0.925	0.690	Respiratory Infection
13	Diet 36 days ACTH 23 days	264	368	0.0275	6.10	—	0.46	0.680	0.490	

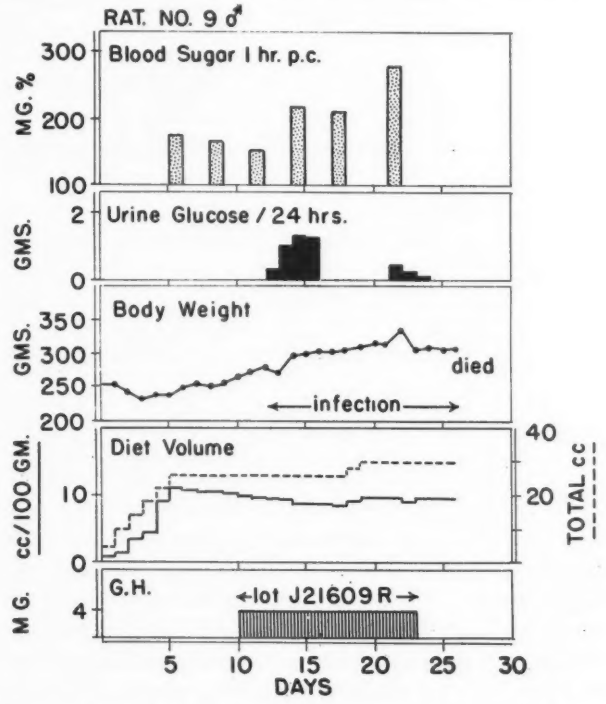

FIG. 7: Temporary glycosuria during growth hormone treatment in a force-fed rat who developed a severe, fatal respiratory infection.

In Figure 8 results are shown which are of significance because of the fact that a more highly purified sample of growth hormone obtained from Dr. A. E. Wilhelmi was used. In this experiment, the animal which was fed 26 ml. of diet throughout, showed only slight glycosuria on 8 mg. of ACTH daily, and brisk glycosuria when 5 mg. of growth hormone were administered for 5 days with the ACTH. The glycosuria promptly dis-

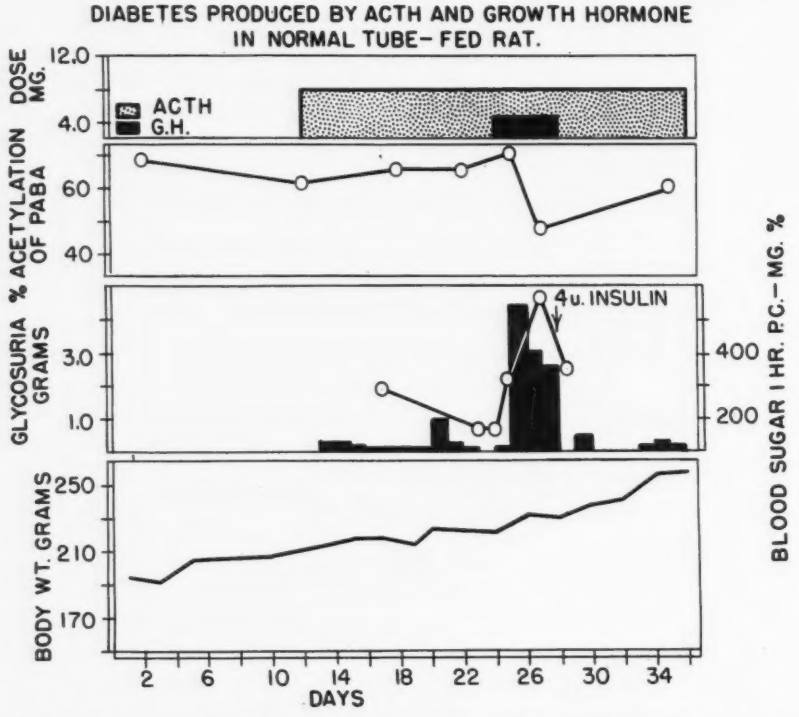

FIG. 8. Temporary hyperglycemia, glycosuria and depression of acetylation of para-aminobenzoic acid during combined treatment with ACTH and a highly purified sample of growth hormone.

appeared on discontinuing the growth hormone. Of added interest is the fact that the per cent acetylation of an injected dose of para-aminobenzoic acid was sharply decreased when the glycosuria developed. Charalampous and Hegsted (1949) have reported that alloxan diabetic rats show an impaired ability to acetylate para-aminobenzoic acid. We have confirmed these results and have in addition found that neither ACTH nor growth hormone treatment influences acetylation except as in this rat, when diabetes results. These studies will be reported elsewhere.

An additional rat (No. 2), whose data are not charted, was studied for 89 days. For the first 30 days the rat, whose initial weight was 293 grams, received 26 ml. of diet and showed no significant glycosuria during a 12 day period of receiving 8 mg. of ACTH, during 4 days of which it also received 5 mg. of growth hormone. During this period, the rat gained little or no weight so that diet was increased over the course of a week to 36 ml. No glycosuria occurred on this diet between the 39th and 55th day although 8 mg. of ACTH daily was begun on the 42nd day. Growth hormone was again started on the 53rd day and continued until the 88th day ACTH being discontinued on the 71st day. During the 18 days of combined treatment, glycosuria ranged between 0.8 and 2.8 grams daily. Glycosuria declined to 0.5 grams or less after ACTH was stopped, but 1 hour post-cibum blood sugar levels of 375, 290 and 225 mg. per cent were recorded. Rapid weight gains were recorded after growth hormone was begun, the final weight being 450 grams. These results are quite comparable to those described in figure 3, rat No. 4.

Gross findings at autopsy in 9 of the rats examined are recorded in Table 1. It will be noted that the adrenals and livers of all the hormone-treated animals and the control rat longest on diet alone (Rat No. 15) were considerably enlarged. Grossly and microscopically, the enlarged livers showed marked fatty infiltration. The fact that hepatic enlargement and fatty infiltration were as apparent in one control rat (No. 15) after 60 days as in the hormone-treated rats, suggests that the high carbohydrate diet may not have been adequate from a nutritional standpoint for long-term use. No other organs showed consistent trends in weight. Microscopic study of most organs was unsatisfactory because of autolysis, the animals usually dying at night.

DISCUSSION

From the above results it is apparent that under these experimental conditions combined treatment with ACTH and growth hormone may result in significant hyperglycemia and glycosuria in the intact rat whereas only minor and irregular glycosuria results from treatment with either hormone alone. Although some hormone-treated rats (No. 1 and 10) exhibited these changes on modest diets, it is interesting that varying degrees of over feeding were necessary in most cases before substantial glycosuria was established. In all cases, however, the diet volumes were well below those which were associated with glycosuria in control force-fed rats. In no case did glycosuria persist after withdrawing hormone treatment. One rat, allowed to eat ad libitum and treated with both hormones for a period of 7 days showed no hyperglycemia or glycosuria. Limitations in supply of hormone precluded further studies along these lines.

Although it is hazardous to attempt to compare results with different lots of hormone in different strains of rats not under comparable experimental conditions, it is interesting to note that the doses of growth hormone which were diabetogenic in these experiments were somewhat less than those found effective by Russell (1951) in increasing the glycosuria of alloxan diabetic rats. This investigator found that 3–5 mg. per 100 grams of an electrophoretically homogeneous preparation of growth hormone were necessary, 1–2 mg. per 100 grams being ineffectual. In our study less than 1 mg. per 100 grams proved effective in rats 3 and 10 (Fig. 5 and 7) while rats 2 and 4 (Fig. 3) showed glycosuria when receiving only slightly more than 1 mg. per 100 grams. The apparent greater sensitivity of the rat to the diabetogenic effect of growth hormone in the presence of adrenal over-activity than in the presence of insulin deficiency is of considerable significance if it can be confirmed under comparable experimental conditions. It would add further support to that derived from the data already presented, that the pancreas is not the only limiting factor in determining the resistance of the rat to the diabetogenic effect of growth hormone. Studies are now in progress to determine the sensitivity of the alloxan diabetic rat to growth hormone and ACTH under conditions comparable to those used here.

The apparent synergism between ACTH and growth hormone found in these experiments does not, of course, represent a new phenomenon. First emphasized by Russell (1940), it has been shown by a number of investigators in different ways that some adrenal hormone is necessary for the full effects of APE or growth hormone on carbohydrate metabolism. Particularly pertinent are the findings of Long, Katzin and Fry (1940) and Long and Lukens (1936) that APE would not sustain diabetes in the adrenalectomized depancreatized animal unless adrenal hormone was present. Park and Krahl (1949) have reported that growth hormone would not influence glucose uptake by the isolated diaphragm of the hypophysectomized-adrenalectomized rat in vitro unless a small amount of adrenal cortical extract were present, an amount which itself did not modify glucose uptake.

The mechanism by which ACTH brings out the diabetogenic effect of growth hormone is not immediately obvious. However, this finding is not inconsistent with the suggestion of Young (1951) that growth hormone is diabetogenic only under those circumstances in which the organism is unable to respond fully to the growth promoting effects of the hormone. Thus the adult dog and cat develop diabetes following growth hormone treatment, while the rat, which continues to grow all its life, the puppy and kitten, and the pregnant or lactating cat or dog do not. In the latter

case of the pregnant or lactating animals, there is in each case a ready site for accelerated protein anabolism, the response being large foetuses and increased milk production respectively. Since the adrenal cortex and growth hormone exhibit antagonistic influences on protein metabolism and growth (Marx, Simpson and Evans, 1943), the diabetogenic response to growth hormone in the ACTH treated rat may be related to the inhibitory effect of ACTH on growth, the animal no longer being able to respond fully to the growth promoting effect of growth hormone. Inspection of the weight curves in the above experiments lends some support for this proposed mechanism. Studies on the effects of other growth inhibiting factors on the response of the rat to growth hormone are being investigated in this laboratory.

The failure to observe permanent diabetes in the rat after combined ACTH and growth hormone treatment contrasts with experiences with growth hormone in the dog and cat. It would appear most likely that the pancreas is the critical factor in this failure of the rat to develop permanent diabetes since the partially depancreatized rat treated with APE may do so (Long, Katzin and Fry). The adrenal cortex, on the other hand, seems to be of greater importance in the initial resistance of the rat to the diabetogenic effect of growth hormone.

SUMMARY

Adult male and female rats force-fed a high carbohydrate liquid diet consistently developed hyperglycemia and glycosuria while receiving both ACTH and anterior pituitary growth hormone. No or inconstant glycosuria resulted on treatment with either hormone alone unless excessive diet volumes were fed. In one rat on a fixed dose of ACTH, glycosuria appeared to vary directly with the dose of growth hormone, whereas the reverse did not appear to be true. The significance of these results is discussed with regard to the comparative roles of the pancreas, adrenal cortex and the capacity for growth in determining the diabetogenic effect of growth hormone in the rat. It is suggested that a level of adrenal activity high enough to prevent the full expression of the growth response to growth hormone may be necessary before a diabetogenic effect of growth hormone becomes apparent, while the high functional reserve of the pancreas may be responsible for the failure to develop permanent diabetes.

Acknowledgements

We are indebted to Dr. Irby Bunding of the Armour Laboratories for generous supplies of ACTH and growth hormone; and to Dr. A. E. Wilhelmi for a sample of highly purified growth hormone.

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MYXEDEMA IN THE RADIOTHYROIDECTOMIZED DOG[1]

R. C. GOLDBERG[2] AND I. L. CHAIKOFF

From the Division of Physiology of the University of California School of Medicine, Berkeley

IN 1934 Trendelenburg, reviewing the early literature, stated that thyroidectomy in the dog results in a pronounced inhibition of skeletal *growth* in addition to other changes. In recent years, however, several reports have appeared (Danowski, Man and Winkler, 1946; Glock, 1949; Binswanger, 1936) indicating that the usual manifestations of hypothyroidism do not develop in dogs in which thyroid deficiency is induced either by surgical excision of the gland or by thiouracil administration. Even though Glock found an increased serum cholesterol as well as histological alterations in the thyroid and anterior hypophysis of thiouracil-treated dogs, she did not observe behavioral or myxedematous changes, growth impairment (puppies), or marked decline in metabolic rate.

A striking study is that of Mayer (1947) who concluded "that beagles, and probably most dogs, do not need the thyroid function under ordinary conditions." After administering propylthiouracil to pure-bred beagle puppies, he failed to observe any impairment in growth rate or bone maturation; moreover, he found no alterations in stature, proportions, hair, mucous membrane, or general behavior suggestive of thyroid deficiency.

That *metabolic* defects do develop in the hypothyroid dog is, however, well established. Thus, an increase in circulating cholesterol was observed, in surgically thyroidectomized dogs, by Chaikoff *et al.* (1941) and by Thompson and Long (1941), and in goitrogen-treated dogs by Glock (1949). An increase in the lipid content of the liver was also found in long-standing thyroid deficiency (Entenman, *et al.*, 1948; Chaikoff, *et al.*, 1948).

The present report deals with the pathology of canine athyreosis. Thyroidectomy was induced by the internal ionizing radiation of I^{131}, a procedure previously used in this laboratory to effect total thyroidectomy in newborn rats (Goldberg and Chaikoff, 1949), in adult rats (Goldberg *et al.*, 1950; Goldberg and Chaikoff, 1950) and in mice (Goldberg and Chaikoff, 1951). This procedure has certain advantages over surgical thyroidectomy because all thyroid tissue, including abberant tissue, can be destroyed.

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[2] Atomic Energy Commission Fellow (National Research Council).

Furthermore, this is accomplished without parathyroid destruction or impairment. Our results show that a condition resembling human myxedema develops in mongrel dogs when the thyroid gland is completely destroyed.

EXPERIMENTAL

Twelve adult, male, mongrel dogs were injected with carrier-free I^{131} (Table 1). Three received a single intraperitoneal injection of 0.2 to 0.5 millicuries per kg., and were sacrificed 2 months later. Two dogs received 2 injections, each consisting of 1.0 millicurie of I^{131} per kg.; an interval of 2 months was allowed to elapse between injections. One of these dogs was sacrificed 1 month, and the other, 2 months after the last injection. Seven dogs received 3 injections of 1 millicurie per kg., with intervals of 2 months between each injection; these animals were sacrificed at intervals up to 1 year after the last injection (Table 1). All dogs were fed *Purina* chow except during short periods when the effects of raw meat on myxedema were studied.

TABLE 1. EFFECT OF I^{131} ON DOG THYROID

Dog number	I^{131} injected		Time between last injection and sacrifice	Residual thyroid tissue
	Millicuries per kg. body wt. per injection	Number of injections*		
			months	
1	0.2	1	2	+
2	0.3	1	2	+
3	0.5	1	2	+
4	1.0	2	1	+
5	1.0	2	2	None
6	1.0	3	2	None
7	1.0	3	5	None
8	1.0	3	12	+
9	1.0	3	12	+
10	1.0	3	12	None
11	1.0	3	12	None
12	1.0	3	12	None

* An interval of 2 months between injections.

The dogs were anesthetized by an intravenous injection of sodium pentobarbital, and exsanguinated. The pituitary glands were fixed in sublimate-formal, and stained by differential methods described elsewhere (Goldberg and Chaikoff, 1952). The thyroid glands, the portion of the trachea adjacent the thyroids, cervical and mesenteric lymph nodes, adrenals, and representative portions of the liver, kidney, stomach, small intestine, lungs, pancreas, heart, and spleen, were fixed in 10 per cent formalin, and stained with hematoxylin-eosin and Masson's trichrome stain. Thyroid gland remnants and other suspicious nodular tissue found in the cervical region from the base of the tongue to the mediastinum (ventral to the prevertebral fascia) was serially sectioned, stained with hematoxylin-eosin, and examined. Frozen sections of the liver and adrenals were stained by Lillie's hematoxylin-oil red 0 technique. Portions of the skin were fixed in formol-alcohol, and sections were stained with hematoxylin-eosin, toluidin blue, and the colloidal iron method of Hale (1946). Best's carmine and the periodic acid-Schiff methods were used for glycogen demonstration.

Chemical analysis of the liver for total fatty acids was carried out by a method described elsewhere (Chaikoff and Kaplan, 1934).

RESULTS

Despite the large doses of I^{131} employed, only 6 dogs were found to be *totally thyroidectomized* (Table 1). Serial sections of the thyroid remnants found in the *other* six dogs (1–4, 8 and 9) revealed various amounts of apparently viable thyroid tissue. This viable tissue was located at the poles and periphery of the glands, the central areas being composed of a core of dense, hyalinized, fibrous tissue which contained abundant yellow pigment granules, occasional fibroblasts and macrophages, and thrombosed vascular channels. The thyroid epithelium appeared active as judged by acinar cell height, colloid content, and high vascularity (Figs. 1 and 2).

The following report deals primarily with the dogs totally thyroidectomized by the I^{131} (dogs 5–7 and 10–12).

Skin—Dogs 10 and 11 were sacrificed one year after the last injection and, at that time, were sluggish and obviously myxedematous (Fig. 3). The skin, particularly that of the neck and legs, was puffy and folded. The hair had become wiry, coarse, and sparse.

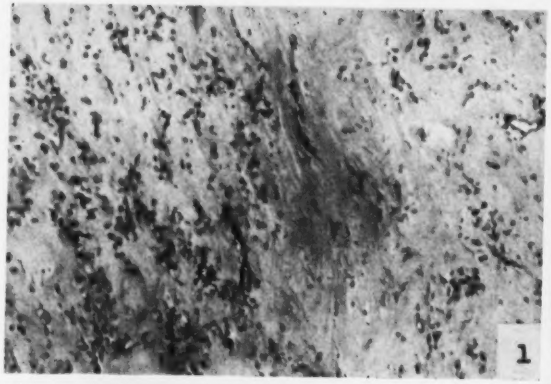
Microscopic examination of skin removed from all totally thyroidectomized dogs revealed the presence of high concentrations of a mucopolysaccharide-like substance (Hale's colloidal iron technique and toluidine blue stain). This substance was removed from the sections by incubation in purified hyaluronidase. Hyperkeratosis of the epidermis, hyperkeratotic plugging of hair follicles and ducts, and epidermal atrophy were also found in the skin of the totally thyroidectomized dogs.

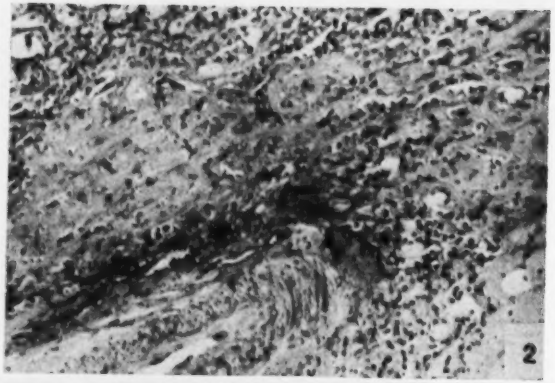
Blood—Examination of blood of dogs 10–12 revealed a 20–30 per cent reduction in red blood count, the cells appearing hypochromic; the marrow was not examined. (No alteration in the hematologic picture was noted in dogs 8 and 9 which were incompletely thyroidectomized.)

Liver—Hepatic fatty change was observed in all 6 dogs. In Dog 5, sacrificed 2 months after the third injection of I^{131}, the liver showed an increased lipid content, particularly in the peri-portal region. The fat appeared as numerous small droplets within the cytoplasm.

In Dog 7, sacrificed 5 months after the last injection of radioiodine, the entire liver was involved in a massive fatty change. The parenchymal cells were distended with large, single, fat globules (Fig. 4). This liver was analyzed chemically and found to contain 35 per cent total fatty acids.

The livers of two totally thyroidectomized dogs (10 and 11) sacrificed 1 year after the last radioiodine injection not only had a high lipid content, but were also rich in glycogen. The liver cells were swollen, and filled with a substance positive to Best carmine (Fig. 5) and to the Schiff reaction.





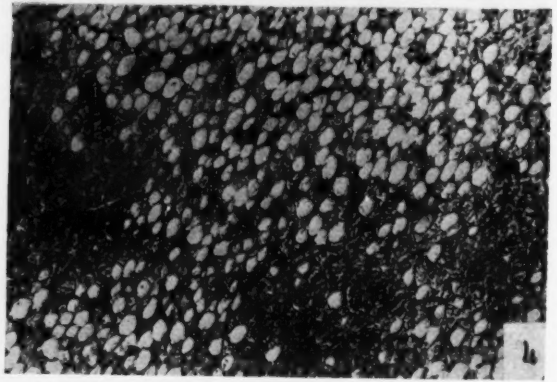


FIG. 1. Central area of remains of thyroid gland from dog sacrificed one year after radioidoine treatment. Dense fibrous tissue, has completely replaced the thyroid parenchyma. Hematoxylin and eosin; ×100.

FIG. 2. Upper pole of thyroid shown in Fig. 1. Note surviving thyroid tissue at tip of superior thyroidal artery and at upper left corner. Hematoxylin and eosin; ×100.

FIG. 3. Myxedematous dog one year after radioiodine treatment.

FIG. 4. Liver of Dog 7, sacrificed five months after radioiodine treatment. The liver has undergone massive fatty change (35 per cent total fatty acids). Hematoxylin and eosin; ×100.

This substance, which was removed by ptyalin digestion (1 hour at 37.5°) (Fig. 6), was presumably glycogen. Lipid in the form of small globules was found in practically all cells. The liver of Dog 12 was quite fatty (20 per cent total fatty acids), and contained but a small amount of stainable liver glycogen (Fig. 7).

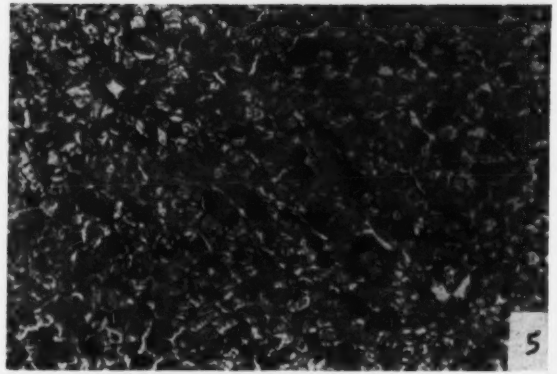
Parathyroid—No pathological alterations were noted in the parathyroids (Fig. 8) other than a slight increase in capsular and stromal fibrous tissue. Tetany was not observed at any time in the I^{131}-treated dogs. Tracheal changes were minimal, i.e., some squamous metaplasia of the lining epithelium, submucosal glandular loss and scarring, and minor cartilaginous alterations.

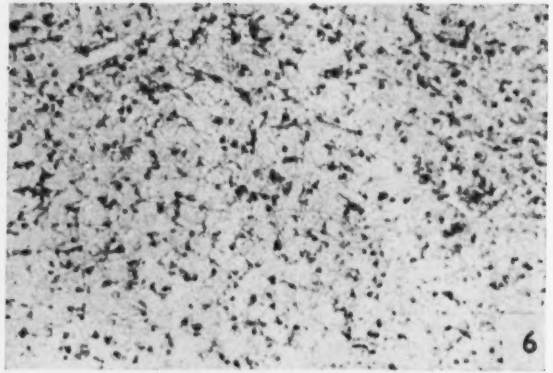
Gastrointestinal Tract and Other Tissues—Dogs 10, 11, and 12 showed slight atrophy of the gastric epithelium, a marked depression in mitotic activity in the intestinal mucosa, involution of cervical and mesenteric lymph nodes, and mucoid degeneration of cardiac, skeletal, and smooth muscle (gastrointestinal). Chronic interstitial nephritis was noted in two dogs, but this is of relatively frequent occurrence in the kidneys of our normal dogs. No pathological alterations were noted in the adrenals, lungs, or pancreas.

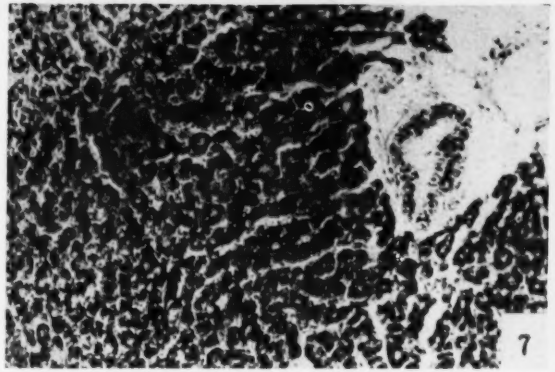
Pituitary Gland—Microscopic examination of the anterior pituitary glands of all 12 dogs revealed marked cytological changes. The glands were characterized by great numbers of extremely large cells (Fig. 9) containing hypertrophied, round nuclei with 1 or 2 fuchsinophilic, hypertrophied nucleoli. The cytoplasm of these large cells was filled with granules that stained positive to aniline blue and negative to the periodic acid-Schiff test and Gomori's aldehyde-fuchsin. Many of these cells (so-called thyroidectomy cells) contained numerous small to large, single, "hyaline" vacuoles (Fig. 10).

The origin of these large cells has not been ascertained with certainty, although they probably arise from beta cells. They were found in greatest numbers in the antero-median portion of the gland, the locus of greatest concentration of the beta, delta, and zeta cells (Goldberg and Chaikoff, 1952). Very little aldehyde-fuchsin positive material was noted in these glands.

Although little or no change was observed in the percentage or morphology of the alpha and epsilon cells in the incompletely thyroidectomized dogs, there was a clear change in the alpha cells of the pituitaries from the totally thyroidectomized dogs. These cells were almost completely degranulated, the rare granulated alpha cell containing only pale-staining, sparse material. The epsilon cells, on the other hand, were well filled with fuchsinophilic granules (Fig. 10). Quantitative estimations of the relative percentages of cells were not carried out.







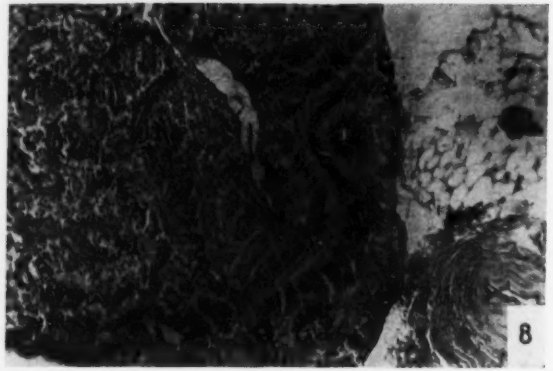


FIG. 5. Liver from totally thyroidectomized dog sacrificed one year after radioiodine treatment. The hepatic cells are swollen and filled with glycogen (black), some of which has diffused away. Best carmine; ×100.

FIG. 6. Section of liver adjacent that shown in Fig. 5. Section incubated prior to staining in ptyalin. Note that material positive to Best carmine has been removed. Best carmine; ×100.

FIG. 7. Liver of Dog 12, sacrificed one year after radioiodine treatment. Note fatty change, most severe in periportal region. The fat appears black in photograph. Hematoxylin and oil red 0; ×100.

FIG. 8. Undamaged parathyroid from totally thyroidectomized dog. Hematoxylin-eosin; ×100.

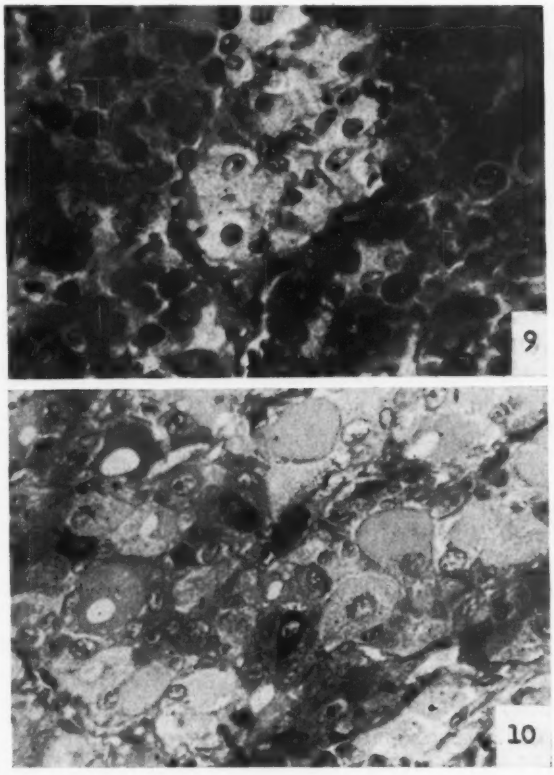


FIG. 9. Anterior pituitary of dog sacrificed one year after radioiodine treatment. Residual thyroid tissue was found in this animal (Fig. 2). A nest of hypertrophied basophilic cells (presumably beta cells) is shown in the center (gray in photograph). Note the well granulated alpha and epsilon cells (black in photograph). Martins; ×500.

FIG. 10. Anterior pituitary of *totally* thyroidectomized dog sacrificed one year after radioiodine treatment. Note vacuolated, hypertrophied "thyroidectomy cells." The 3 black cells are epsilon cells. The alpha cells are degranulated. Martins; ×500.

DISCUSSION

The conclusion that thyroid function is of no importance to the dog is not borne out by the results reported here. The obvious sequelae of acute hypothyroidism observed in man and other species—myxedema and behavioral changes—were evident in our dogs lacking thyroid function. In addition, pathologic alterations in blood, gastrointestinal tract, liver, muscle, lymphoid tissue, and particularly in the pituitary gland, clearly reflect the total lack of thyroxine in the dog.

The dog may require only minute amounts of the thyroid hormone for

the maintenance of an apparently normal state. We have observed that when radio-thyroidectomized dogs are fed a meat-free but adequate diet, the physical symptoms of hypothyroidism are exaggerated, whereas when raw meat is fed, the myxedematous manifestations are diminished. It is possible that the small amounts of thyroxine contained in the meat alleviate some of the myxedematous changes. Griesbach and Purves (1943) have observed a somewhat similar phenomenon in anterior pituitary of thyroidectomized rats. Thus, complete alpha cell degranulation occurs in the pituitaries of thyroidectomized rats maintained on a meat-free diet; but when these rats are fed a diet containing meat meal, the alpha cells remain granulated. These investigators attribute this effect to the small amounts of thyroxine in the meat meal.[3]

The vacuolated, hypertrophied cell (the so-called thyroidectomy cell) has been observed in the anterior pituitary of completely and incompletely thyroidectomized dogs. However, in those shown to be completely deprived of thyroid tissue, the alpha cells were, in addition, degranulated. The epsilon cells, on the other hand, were apparently unaffected. The failure of previous investigators to find alpha cell degranulation in dogs may be ascribed either to incomplete thyroidectomy or inability to distinguish between alpha and epsilon cells, both of which were termed acidophils with the older staining methods.

SUMMARY

1. Twelve dogs were injected with various doses of I^{131} and sacrificed at intervals of 1 to 12 months later. In 6 of these dogs, all thyroid tissue had been destroyed by the ionizing radiation.

2. Myxedema and behavioral changes characteristic of hypothyroidism were observed in 2 of the 3 completely thyroidectomized dogs kept for one year after the injection.

3. The following other changes were observed in completely thyroidectomized dogs: an increase in the acid polysaccharide content of skin; hyperkeratosis and epidermal atrophy of skin; anemia; increased liver fat; muscle degeneration; lymph node involution; and atrophy of gastrointestinal tract.

4. "Thyroidectomy cells" were found in the anterior pituitary of both subtotally and totally thyroidectomized dogs. Alpha cell degranulation was found in *only* the totally thyroidectomized animal.

5. Radiation damage to the parathyroids and trachea was insignificant in all I^{131}-treated dogs.

[3] 0.05 to 0.10 γ thyroxine per 100 gm. body weight per day will effectively prevent total alpha cell degranulation in the thyroidectomized rat.

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TRANSIENT OXYTOXIC DEPRESSION OF IMMATURE GUINEA PIG FED ON GRASS (*BRACHIARIA PURPURASCENS* (RADDI) HENRY)

J. PEREIRA, JR.

Department of Animal Physiology, Instituto Biológico, São Paulo, Brazil

WHILE engaged in the "in vitro" assessment of oxytoxic activity of posterior pituitary extracts, we observed a progressive decline in strength besides an increasing sluggishness of the responses of the young guinea pig's uteri—in animals of the same stock and comparable body weights— to doses of a potent posterior pituitary extract.

A careful checking of the experimental conditions showed that a very common panic-like green fodder (*Brachiaria purpurascens* (Raddi) Henry) had been substituted for the abitual kale leaves (*Brassica oleracea* L) of the ration. The grass, formerly intended as bedding, was eaten apparently as greedily as kale, together with the basic supplement of grain polishings always available "ad libitum."

Some normal, immature guinea pigs, besides the well known causes of variability in oxytoxic testing, like season, age, nutritional state, hormonal treatment or deprivation, are not suitable for oxytoxic testing.

We shall try to evidence an unsuspected cause of variation of oxytoxic activity bound to the "greens" in the diet of the immature guinea pig.

MATERIAL AND METHODS

Young, virgin guinea pigs about 30 days old, from a much inbred colony, were distributed in groups and raised on *Brachiaria*, kale or a mixture of both greens, in wire bottom cages. The vitamins and mineral salts of grain polishings were liberally supplied as a basic supplement, enriched for three of the groups with meat and casein.

The supplement as routinely furnished to the colony had the following percentile composition: wheat polishings 20.2, tikitiki 20.2, medium grade corn meal 22.6, peanut cake 11.3, corn polishings (30 per cent protein) 22.6, meat meal 2.3, bone meal 0.7, crude salt 0.4. All dietary additions were made at the expense of the corresponding weight of this supplement.

The animals, maintained at about 22° C., were weighed thrice a week; vaginal smears were made in the week days and interpreted according to Young (1936–37).

A press juice of the grass, evaporated to dryness at 105° C. for 2 days to destroy all heat labile substances was reduced to a dark brown powder to be added to the basic supplement at 4.2 per cent, this being equivalent mostly to the mineral salts of 200 g. of fresh grass.

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The experiment lasted a year and a half, but we are here concerned with the oxytoxic tests done on 76 guinea pigs in the dry months, May and June, 1950 and May, 1951. Two animals were discarded: one after a sudden stretch of the uterus while in the apparatus; the other, for having only 142 g. of body weight.

To test simultaneously the uteri from different animals of similar body weight on distinct diets, we assembled two, and later three, well balanced frontal writing levers with an arm ratio of 6:1, loaded with 0.5 g. symmetrical and equidistant to the point of application of the muscular traction. The weight might be slipped freely to any point of the lever arm with a corresponding magnification of load. The overload of 0.5 g. $\times 6$ was also used.

After a few experiments, the volumes of the thyroid glands, the ovary (and tubes) and the uteri and fundus, all trimmed from adjacent tissues, were successively determined in a volumometer constructed after Brudick (1941), filled with Ringer solution in thermic equilibrium with the water bath, thus minimizing desiccation and temperature changes of the organs. One of the uteri, both ovaries and thyroid glands were dropped in the fixing solution of 10 per cent formaline and 5 per cent ethyl alcohol, and the sections stained with hematoxylin and eosin. Two out of about 200 guinea pigs, had an uterus simplex with normal, paired ovaries.

The calcium titrated Ringer solution (Burn, 1937) stored at room temperature as a 33 times concentrated solution, with sodium bicarbonate and dextrose added upon dilution, was prepared just before each test.

The uteri were tied to the oxygen tubing and immersed in a well oxygenated Ringer bath at 37° C. After each dose the Ringer was rapidly syphoned out twice in the 20 to 40 minutes the preparations were allowed to rest.

The commercial solutions from the same package, of total posterior pituitary preparation, 10 I.U./cc., histamine hydrochloride 0.5 mg./cc., and oestrone in sesame oil 1 mg./cc., of well reputed brands, were suitably diluted, ampulled and tyndalized for use. The millesimal dilution of the I.U. of posterior pituitary solution, as rediluted just before the test is called milli I.U. From 0.1 to 0.5 cc. of the test solutions are employed so as to cause the least dilution and pH alteration of the Ringer bath.

The maximal oxytoxic activity in millimeters in the record to the smallest dose of stimulus for each of the two or three simultaneously tested uteri is chosen as best expressing the muscular strength. In fact, the liminal response is sometimes very discrete and is not elevated in proportion to higher doses.

EXPERIMENTAL

In the upper part of Figs. 1 and 2, each symbol represents the maximal height of oxytoxic response obtained with the test substances. In the lower part, the same symbols stand for the dose of corresponding oxytoxic drugs. The tailed black dots represent the actual body weight on test day, while the direction and length of the tail from the starting weight, means the gain or loss in weight.

The uteri are generally thin and whitish or slightly pink, being both macro and microscopically normal. The cellular pattern of the vaginal smear does not reflect with fidelity the capacity of oxytoxic activity; in the maturing females of Fig. 1, on grass, the vaginal picture of stage 1-b late was the commonest; on grass and kale or on kale only, even in very

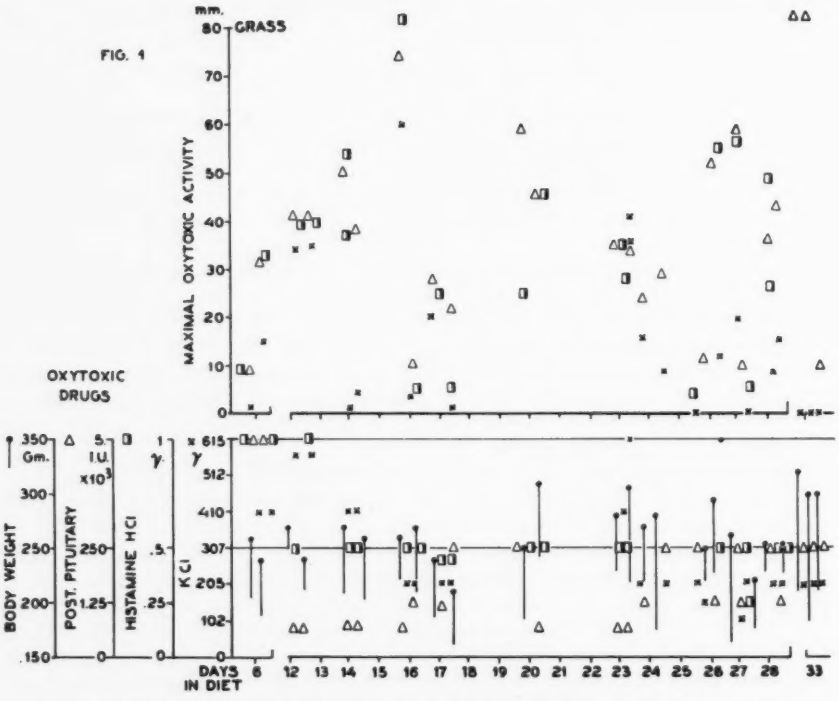

FIG. 1. Immature guinea pigs raised on *Brachiaria* grass and basic supplement.

Upper part: Maximal oxytoxic responses in millimeters in the record to total posterior pituitary solution, histamine hydrochloride and potassium chloride.

Lower part: Each symbol stands for the corresponding dose of each oxytoxic drug. The length of the "tail" in body weight symbols shows the gain in weight. It is to be observed the parallelism of responses to posterior pituitary solution and histamine hydrochloride, with a weaker action of potassium chloride. As the animals grow mature the oxytoxic activity tends to be elicited with lower doses of drugs but not with higher contractions.

young guinea pigs of about 200 g., as shown in Fig. 2, the vaginal smear of stage III, with abundant invading leukocytes, is predominant after the second or third smear.

Hemorrhagic follicles or corpora lutea, in no instance have been found either macroscopically or in sections.

Fig. 1 shows the maximal oxytoxic responses of pairs of differently supplemented grass fed animals, as elicited in 25 guinea pigs, through the solutions of posterior pituitary, histamine hydrochloride and potassium chloride, in an attempt to reach by different ways the contractile mechanisms of the smooth muscle, as influenced by the diet.

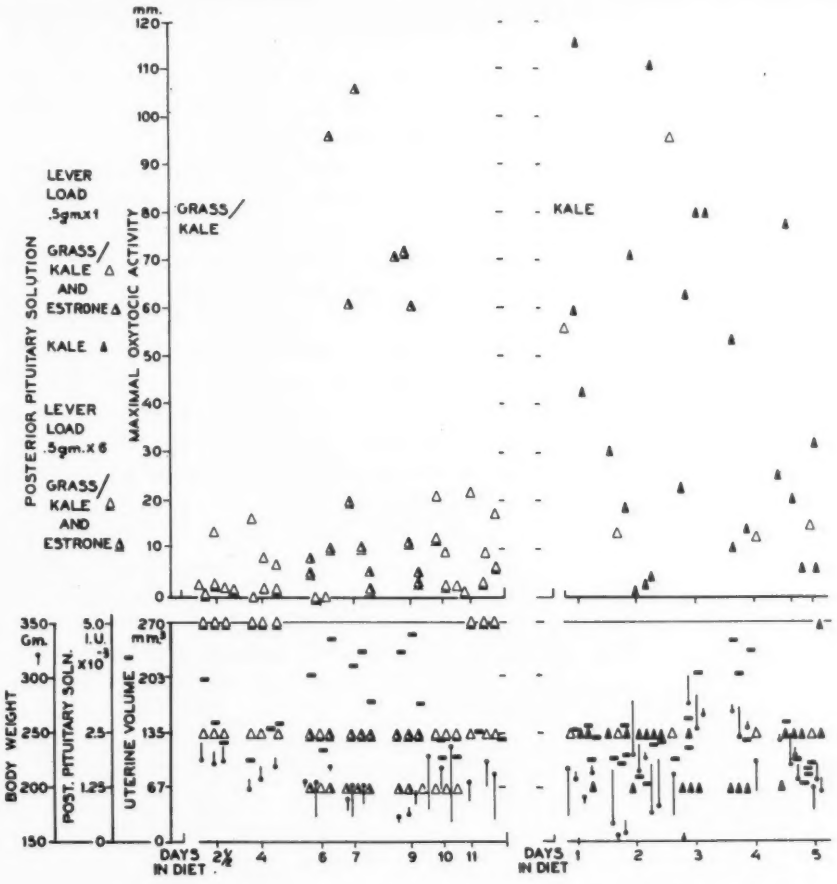

FIG. 2. Enhanced oxytoxic activity of immature guinea pigs after the withdrawal of *Brachiaria* grass.

Upper part: Maximal oxytoxic activity in millimeters in the record to posterior pituitary solution. Action of estrogen and of an overload of 0.5 gm. ×6.

Lower part: Each symbol corresponds to the dose of posterior pituitary solution giving the maximal response, and the action of estrogen, and overload. The length and direction of the "tail" in body weight symbol represents the weight gain or loss in diet. The horizontal bars represent the uterine volumes.

The *left segment* shows the inadequacy of kale to counteract the oxytoxic depressing action of *Brachiaria*. This depression can be overcome in most cases through small doses of estrogen that bring a sharp increase in uterine volume, but not after overload. The *right segment*, shows animals under kale diet (5 under grass). It is to be observed that the awakening of oxytoxic activity is both immediate and not dependent on body weight, or uterine volume, and is not hindered by acute weight loss.

The animals have been raised throughout on grass and basic supplement; the number of days of diet is represented by the number of days in which the animals, though continuing on grass, did not receive the basic supplement or received it both plain or enriched either with 5 and 10 per cent meat meal or with 5 per cent commercial casein.

The modification of the supplement or its withdrawal under grass feeding can not overcome the oxytoxic inhibition, as shown by the generally weak and, in more than one third, sluggish responses obtained even when the animals were putting weight continuously for about two months: they were tested from about 250 g. up (mean weight: 266.4 g.) The mere statement of the contraction height does not reveal its sluggishness, since in 7 animals more than 10 minutes were necessary for the maximum to be attained, irrespective of their body weights.

It is to be observed that from the 6th to the 33th day there is a tendency of the oxytoxic activity to progressively escape from the grass depression; this escape should not be measured only by a higher contraction, but also and chiefly through a generally smaller test dose of the drugs employed.

After 33 days of the diet 3 animals (about two months old) were simultaneously tested. Two animals had so matured as to exhibit thick, congested uteri, characteristic of oestrus; the vaginal smear of one of them showed stage 1-b late; the other, stage III. Both had given the highest response to the pituitary solution; potassium chloride was here, as in some other animals, deprived of action.

There is generally a close parallelism between the oxytoxic activity of the posterior pituitary principle and histamine hydrochloride, notwithstanding the latter drug being admittedly active on a "link" closer to the contractile mechanism (cf. Fischer, 1944), than the pituitary oxytoxic principle. Potassium chloride is more irregular in action and frequently inactive when a response is obtained with the other drugs: the elevation of the dose is prone to produce relaxation.

In eight animals a progressive development of tonus after each dose inhibited the full relaxation, while the companion uterus of another animal exhibited its own reaction to the drugs.

From Fig. 1 it is apparent that the strength (and also velocity, not shown in the graph) of the uterine muscle contraction in immature *Brachiaria*-fed guinea pigs, is fairly independent of the body weight.

In order to find out (Fig. 2) whether this inhibition could have an anatomic counterpart not readily apparent to gross or microscopic inspection, the volumes of the uteri (horizontal bars), ovaries and thyroid (not shown) were determined in quite young animals (about 30 days old) raised on *Brachiaria* plus basic supplement. These animals had been separated into

three lots, viz., 14 (mean body weight: 211 g.) were changed to a liberal diet of grass and kale; 25 (mean body weight: 215.6 g.) were transferred to a kale diet and 12 remained on the grass diet. This last lot is scattered in both segments of Fig. 2. All three groups received the basic supplement.

Powdered grass juice, the proportion of 4.2 per cent, equivalent to 200 g. of fresh grass, was added to the basic supplement of 7 animals on grass and kale diet and 5 ones on kale only. This addition did not cause any alteration in the oxytoxic response.

The left segment of Fig. 2 shows maximal responses of uteri of three simultaneously tested animals on grass and kale (grass not distinct), under overload and oestrogen.

It is apparent that under *Brachiaria*, even with added kale, the immature animals have a very poor capacity of reaction to the posterior pituitary solution.

To stretch the uterine muscle, so as to produce a longer and stronger fiber, the 0.5 g. load was transferred to the extremity of the lever producing a 0.5 g.$\times$6 overloading. This increase in strength is already known to be peculiar to striated muscles and to strips of pregnant guinea pig's uterus as verified by Newton (1933). No enhancing effect but on the contrary, a greater depression has been obtained, thus confirming altogether Simeone's observation (1935) for the non-pregnant cat uterus, i.e., the rhythmic activity has no proportionality to the tension thereto applied.

To ascertain if a more developed uterus, possibly rid of the grass depression could be obtained, 8 animals (left segment, Fig. 2) were tested respectively on the 6th, 7th and 9th days of this mixed "greens" diet. They were injected with the low subcutaneous dosage of 2.5 gamma of oestrone in oil, either 48 or 96 hours previously. The enhancing effect of the injected estrogen is evidenced by the strong oxytoxic response, which in 5 of the animals attained the level shown in Fig. 1 or even a higher level, irrespective of the conspicuous uterine thickening provoked in all of the animals, as shown in the lower part of the graph. It is worth being recalled that the animals studied in Fig. 1 were more fully mature.

Three of the injected guinea pigs did not give an enhanced oxytoxic response after this dosage of estrone. Inasmuch as some otherwise normal guinea pigs are known to have a higher threshold to estrogens (Young, Dempsey, Myers and Hagquist, 1938; Wilson and Young, 1941), this seemed not to be the case here for the uterine volume was sharply augmented in all animals under estrogen, with individual variations. Even under estrogen the capacity of good oxytoxic reaction with overloaded levers showed itself only in one animal, tested on the 9th day, which had been accidentally injected twice with 2.5 gamma of estrone 96 and 48 hours before the test.

It is obvious that the oxytoxic depression conditioned by the grass diet and sustained by grass and kale can generally be overcome by low doses of estrogen, intended to act on the uterine muscle without interfering with other endocrines, namely the hypophysis or the thyroid glands. This suggests the green fodder to be one of the extrinsic causes of the known threshold variability to estrogen as shown by guinea pigs.

The right segment of Fig. 2 shows the speed of reversal of the grass conditioned depression of oxytoxic activity through its mere displacement from the diet. Here, equally immature guinea pigs raised on grass and basic supplement, after being transferred to kale with basic supplement, show immediate enhancement of oxytoxic activity. The animals passed from grass to kale in successive days. This allowed the simultaneous testing of three animals at different stages in the course of their kale diet.

The kale diet, while readily accepted, has systematically induced a weight loss in these trials of short standing.

It is apparent from the right segment of Fig. 2, that independently from immaturity, weight loss or uterine development, the oxytoxic activity is here much higher than in normally growing, grass-fed guinea pigs.

The oxytoxic depression observed in the grass-fed guinea pig is not conditioned by any anatomic or histologic alteration, for all tissues looked normal. This is stressed even more by the rapid heightening of oxytoxic activity under kale.

Though inadequate for growth, the kale diet, by subtracting the uterine muscle from the depressive action of the grass feeding, strongly mimetizes the action of the estrogen previously referred to, thus conditioning the intensity of the "in vitro" oxytoxic activity of immature guinea pigs to the diet greens.

The better oxytoxic activity obtained under an exclusive kale feeding, in quite immature guinea pigs, is largely independent either of the body weight or weight loss or even from the uterine development, as seen in both segments of Fig. 2.

In fact, it is apparent with animals under grass and kale that a full oxytoxic response is only obtained when the uterine volumes are above 200 $mm.^3$ as observed under estrogen priming. Animals with uteri from 130 $mm.^3$ up to 178–198 $mm.^3$, are quite irresponsive.

In contradistinction, under kale a satisfactory oxytoxic reaction can be obtained with uteri of about 130 $mm.^3$ and occasionally even smaller, notwithstanding the fall of the body weight after diet change.

Not all immature animals under kale are apt to react to posterior pituitary solution even as high as 5 milli I.U., and when a low and sluggish contraction is obtained here also it is of no avail to try a higher dose.

In three instances, in this group of animals emerging from the oxytoxic depression a rapid fatigability has been found with uteri giving a good initial response not to be reproduced successively even with larger doses. The enhancing responses to repeated doses are sometimes obtained in any diet, as already known.

The absence of uterine development, or the corresponding histology, does not favor kale as a source of ingested estrogen.

DISCUSSION

The appraisal of the influence of *Brachiaria* as part of the immature guinea pig's diet, on the type of oxytoxic response is here presented. The grass diet conditions a higher uterine threshold, and even inactivity to oxytoxic agents, with weaker and generally more sluggish type of uterine contraction, without any evidence of malnutrition.

The quick reversal of the grass feeding depression of oxytoxic activity through a kale diet, though inadequate for growth, suggests the presence in the grass of inhibiting substance(s) quickly destroyed or eliminated by the organism, thus allowing the obtention of stronger uterine contractions, when the immature animals are switched from the grass to the kale diet. A uterus smaller (in volume) than the ones obtained under grass and estrogen is not a hindrance to a better oxytoxic response.

The mineral salts, vitamins and proteins of the basic supplement, even when enriched with the solids of the press juice of the grass, have no effect on the oxytoxic depressive action of *Brachiaria* on guinea pigs growing well on this diet.

A similar depression of oxytoxic activity in the rabbit was obtained by Makepeace, Corner and Allen (1936) by the use of progesterone.

In our experiments, where such a hormonal factor was not introduced, it is unlikely that it might be secreted by the ovaries in augmented quantities, because a) our animals are immature and, as in Fig. 2, very immature; b) in no instance has a corpus luteum or, at least, a ripe follicle been observed.

By the undernutrition of mature guinea pigs in restricted diets with a 20–30 per cent weight loss in 2 weeks, Stephens and Allen (1941) have obtained an ovarian picture similar to that obtained after hypophysectomy, responding well to an anterior pituitary preparation, but not to thyroxin.

The very acute weight loss in the immature guinea pigs under kale is not comparable to the weight loss of the mature undernourished guinea pigs. The ovarian, thyroid and uterine pictures are here, as on grass, essentially normal, as evidenced by the frequently higher and more rapid uterine contractions verified on kale and after estrogen priming.

The enhanced oxytoxic responses of immature guinea pigs under kale are not dependent on any peculiar uterine development in volume, thus making inconsistent the hypothesis of augmented estrogen action, either ingested with kale or through ovarian hyperactivity.

The growth of the guinea pigs under *Brachiaria*, as contrasted with the weight loss under kale diet, is an evidence against the influence of nutritional state or of body growth "per se" on the threshold for oxytoxic reactivity.

The growing grass has, in fact, growth factors for guinea pigs and rats, as first shown by Kohler, Elvehjem and Hart (1958). Actually, the kale substitution for grass is immediately followed by a weight loss, suggesting the absence of growth factors in kale. Notwithstanding this, better responses are obtained under kale thus showing the relative degree of independence of the oxytoxic activity in immature guinea pigs from age, season, etc., so stressing the importance of the nature of the "greens" in diet.

Summary

Immature guinea pigs (mean body weight: 266 g.) raised on *Brachiaria* grass exhibit a marked and transient depression of oxytoxic activity "in vitro," notwithstanding the good body growth obtained.

The supplementation of this grass with grain polishings, either enriched with animal protein or the solids of the grass juice, or even the elongation of the muscle fiber does not overcome the depressive state of the uterus. A rapid reversal of the inhibition can be brought about either through small doses of estrogen in grass-fed animals, bringing the uterine volume to above 200 $mm.^3$, or through the substitution of kale for grass even in quite immature animals: mean body weights 211–215.6 gm.; uterine volumes under 130 $mm.^3$

The absence of uterine development under kale precludes the action of augmented quantities of estrogen either endogenous or exogenous.

There are no gross or fine anatomic alterations through undernutrition, in the ovaries, uteri or thyroid glands, as stressed by the rapid disappearance of the grass conditioned depression, when this "green" is substituted for kale. This suggests the presence in the ingested *Brachiaria*, of oxytoxic inhibiting substance(s), mimetizing the already known action of progesterone, but very quickly eliminated or destroyed by the organism, and whose action can be slowly overcome as the guinea pig grows mature.

The oxytoxic response in the immature guinea pig does not seem to be related to body weight (age), vaginal smear, or uterine development (volume), but rather to the nature of the "green" in diet, this being, possibly, one of the causes of threshold variation to injected estrogen in this species.

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I^{131} DISTRIBUTION IN NORMAL AND HEMITHYROIDECTOMIZED RATS TREATED WITH DESOXYCORTICOSTERONE ACETATE

JOSEPH B. BOATMAN, CLEM RUSS, JOSEPH H. SUNDER, ANDREW KONNERTH AND CAMPBELL MOSES

From the Addison H. Gibson Laboratory, the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

THE similar biological effect on membrane and cellular permeability of desoxycorticosterone acetate and hyaluronidase has been reported (Opsahl, 1949a), (Opsahl, 1949b), (Seifter, Baeder, and Begany, 1949). Recent studies have shown that several endocrine glands exhibit widely varying concentrations of radioiodine under various experimental conditions (Franke, Boatman and George, 1951), (Boatman and Moses, 1951a), (Boatman *et al.*, 1951). The study here reported was conducted to determine whether the variation in the ability of these tissues to concentrate radioiodine was altered by the administration of low levels of desoxycorticosterone acetate (DCA).

METHODS AND PROCEDURE

Holtzmann-Rolfsmeyer female rats, weighing between 210 and 230 grams, were randomly divided into 4 groups of 16 animals each. The weight of each animal was within twice the standard deviation of the mean weight of all animals. All animals were housed under identical conditions and were maintained on tap water and Rockland rat ration.

Groups I and III rats were hemithyroidectomized by removing the right lobe and half the isthmus of the thyroid gland under light ether anesthesia. Groups II and IV were sham-operated, the thyroid gland exposed and palpated under light ether anesthesia and the incision closed. Ten days following the surgical procedure, the animals were placed on the following regimen.

Groups I and II rats were injected intramuscularly three times weekly with 0.2 ml. of sesame oil, N. F., and Groups III and IV rats were injected three times weekly with 0.2 ml. sesame oil containing 1.0 mg. desoxycorticosterone acetate (DCA)* for a total of 3.0 mg. of DCA per week per rat in Groups III and IV. The sites of injection were changed at each injection time to avoid the accumulation of DCA or sesame oil at one location.

All animals were sacrificed after 30 days. Three hours before sacrifice each rat received 200 microcuries of intraperitoneal I^{131} as carrier free NaI in a physiological saline solution. At sacrifice under ether anesthesia, heart blood was collected directly into cold heparinized test tubes, the animal exsanguinated, and the pituitary, thyroid and ad-

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* *Percorten*, Ciba Pharmaceutical Products, Summit, New Jersey.

renals quickly removed, rinsed in saline and weighed on a torsion balance to the nearest tenth of a milligram. One half of each organ was pooled with others from the same group, and the remaining half weighed, wet with $AgNO_3$ solution and ashed at 300° C. under aluminum caps and assayed for radioiodine I^{131} (Boatman and Moses, 1951a).

The pooled aliquots were chilled in dry ice, homogenized under trichloroacetic acid, and the radioiodine I^{131} content of the acid soluble and insoluble fractions determined under identical, controlled conditions, followed the methods described by Wolff and Chaikoff (1948) and Albert and Lorenz (1951). The radioactivity of the acid soluble fraction was considered to contain the inorganic iodine portion, while radioactivity of the insoluble fraction was considered to represent principally the organically bound iodine components of the tissue. In a preliminary study, following the addition of I^{131} to the trichloroacetic acid, less than 2.0% of added I^{131} was found in the acid insoluble fraction of the tissues of normal, untreated rats.

Hematocrit determination was made on the blood of each animal in a Wintrobe hematocrit tube and pooled blood from each group was used for the determination of whole blood, plasma and erythrocyte radioiodine I^{131} (Boatman and Moses, 1951b), and for the determination of the radioiodine I^{131} content of the trichloroacetic acid soluble and insoluble fractions of the plasma.

The per cent of administered activity in each fraction of tissue assayed was calculated in terms of the total organ weight. In the case of whole blood, 5% of the total body weight was used as an estimate of whole blood volume, and the plasma volume was calculated using the average hematocrit value. The concentration of I^{131} in each organ was expressed in terms of radioiodine I^{131} concentration ratios as previously described (Boatman and Moses, 1951a). The per cent of administered activity found in an organ divided by that organ's per cent of total body weight is termed the concentration ratio. This value reflects the ability of an organ to concentrate the isotope, and values so expressed are considered to be independent of variations in the size of organs or of the animal.

RESULTS

No significant changes were observed in gross weight or in the per cent of body weight of the organs examined (Table 1).

The DCA treated groups showed a consistently higher per cent of whole blood I^{131} in the erythrocyte than the corresponding control groups, either hemithyroidectomized or sham operated, as well as a greater per cent of administered activity in the estimated total blood volume of the DCA groups. Hemithyroidectomized animals showed a lower per cent of total

TABLE 1. WEIGHT IN GRAMS AND MEAN PER CENT OF BODY WEIGHT (BW) OF ORGANS OF HEMITHYROIDECTOMIZED (GROUPS I AND III) AND SHAM OPERATED (GROUPS II AND IV) RATS RECEIVING DESOXYCORTICOSTERONE ACETATE (DCA) OR SESAME OIL

	Sesame oil				DCA treated			
	Group I		Group II		Group III		Group IV	
	mg.	% BW	mg.	% BW	mg.	% BW	mg.	% BW
Pituitary	10.9	.0047	13.0	.0057	12.8	.0054	13.2	.0057
Thyroid	6.9	.0030	15.7	.0069	7.8	.0033	17.7	.0076
Adrenals	51.4	.0222	54.6	.0240	51.4	.0215	54.3	.0234

TABLE 2. DISTRIBUTION OF ADMINISTERED I^{131} IN THE BLOOD OF HEMITHYROIDECTOMIZED (GROUPS I AND III) AND SHAM OPERATED (GROUPS II AND IV) RATS TREATED WITH DESOXYCORTICOSTERONE ACETATE (DCA) OR SESAME OIL

	Sesame oil only		DCA treated	
	Group I	Group II	Group III	Group IV
Estimated Whole Bld. Volume	10.83 ml.	11.12 ml.	11.97 ml.	11.59 ml.
Hematocrit	47.4%	45.7%	40.2%	44.9%
% Adm. Act. in Whole Bld.	7.63%	6.00%	10.61%	10.15%
% Adm. Act. in Plasma	5.89%	3.92%	7.22%	5.93%
% Adm. Act. in RBC (Differ.)	1.74%	3.08%	3.39%	4.22%

blood I^{131} in the erythrocyte than did the sham operated groups, either with DCA or sesame oil only, confirming an earlier report of this event in hypothyroid rats (Boatman and Moses, 1951b) (Table 2).

There was observed an increase in I^{131} concentration ratios for all tissues from animals receiving DCA when compared to animals treated with sesame oil only.

In the non-DCA, sesame oil treated Group I, the hemithyroidectomized animals exhibited an increased I^{131} concentration ratio in the pituitary and remaining thyroid tissue and a slight decrease in the adrenals when compared with the non-hemithyroidectomized, sesame oil treated animals (Group II).

In the pituitary and thyroid tissue (Group I) there was found an increased percentage of inorganic I^{131} content, and a decreased percentage of organic I^{131}. In the adrenal (Group I) there was found a decreased percentage of inorganic I^{131} compared to the non-hemithyroidectomized animals (Group II).

The pituitary tissue of the DCA treated, hemithyroidectomized animals (Group III), showed lower I^{131} concentration ratios than the DCA treated, non-hemithyroidectomized group (Group IV). This was accompanied by decreased percentage of inorganic I^{131}.

The thyroid tissue of the two groups of DCA treated animals (Groups III, IV) showed similar relationships as the non-DCA groups, (Groups I, II) except that the I^{131} concentration ratios were greater in the DCA treated animals than in the other animals.

The adrenal tissue of the DCA-treated, hemithyroidectomized (Group III) showed a markedly decreased percentage of inorganic I^{131} with an increased percentage of organic I^{131}, and greatly increased content of total I^{131} present, expressed as the I^{131} concentration ratio.

It should be noted that both pituitary and adrenal tissue of hemithyroidectomized rats treated with DCA (Group III) reversed the percentage of inorganic I^{131} to organic I^{131} percentage, and assumed a ratio of distribution similar to that consistently seen in thyroid tissue of all groups, where the organic I^{131} percentage content exceeded that of the inorganic content (Table 3).

TABLE 3. CONCENTRATION RATIOS OF I^{131} (CRI^{131}) IN TISSUES OF HEMITHYROIDECTOMIZED (GROUPS I AND III) AND SHAM OPERATED (GROUPS II AND IV) RATS TREATED WITH DESOXYCORTICOSTERONE ACETATE (DCA) OR SESAME OIL

CRI^{131}	Sesame oil only		DCA treated	
	Group I	Group II	Group III	Group IV
Pituitary:				
total	.814	.545	.357	.487
acid-soluble	.662	.390	.089	.323
(% of total)	(81.2%)	(67.4%)	(24.8%)	(66.3%)
acid-insoluble	.153	.188	.269	.164
(% of total)	(18.8%)	(32.6%)	(75.2%)	(33.7%)
Thyroid:				
total	255.478	193.727	428.192	254.807
acid-soluble	47.115	19.968	60.631	24.566
(%of total)	(18.4%)	(10.4%)	(14.2%)	(9.6%)
acid-soluble	208.377	367.562	367.562	230.241
(% of total)	(81.6%)	(89.6%)	(85.8%)	(90.4%)
Adrenal:				
total	.519	.556	1.408	.510
acid soluble	.319	.443	.502	.319
(% of total)	(61.2%)	(74.6%)	(30.3%)	(62.4%)
acid-insoluble	.202	.151	1.054	.192
(% of total)	(38.8%)	(25.4%)	(69.7%)	(37.6%)
Plasma:				
total	.169	.122	.120	.148
acid-soluble	.163	.119	.117	.145
(% of total)	(96.4%)	(97.5%)	(97.5%)	(98.0%)
acid-insoluble	.006	.003	.003	.003
(% of total)	(3.6)	(2.5%)	(2.5%)	(2.0%)

DISCUSSION AND CONCLUSIONS

Surgically reducing the amount of thyroid tissue produces moderate hypothyroidism and may result in hyperactivity of the remaining thyroid gland. The increased I^{131} concentration ratios of the remaining thyroid tissue found with or without the administration of DCA in these experiments supports this view.

From the data presented it appears that small amounts of DCA ad-

ministered to normal and hemithyroidectomized rats may alter the distribution of carrier free I^{131} in the blood, pituitary, thyroid and adrenals. In general, the effect is one resulting in an increased content of radioiodine in those tissues and in the red cell fraction of the blood. It is assumed that these alterations are related to the DCA injected.

An increase of the I^{131} concentration ratios could be expected if the excretion of I^{131} was decreased. Existing evidence supports the view that the behavior of the iodine ion corresponds closely to the chloride ion in distribution. An examination of the blood data from this study indicates that the whole blood, plasma and erythrocyte I^{131} content were about 30% higher in the animals receiving DCA. Concentration ratios of the inorganic and organic fractions in these animals remained essentially the same, representing a quantitative increase in I^{131} levels in the blood. An increase of approximately 35% and 45% in the thyroid and adrenal I^{131} concentration ratios respectively, of animals receiving DCA, could reflect a depressed rate of I^{131} excretion during the 3 hours prior to sacrifice. However, the pituitary I^{131} concentration ratios of the non-DCA animals were approximately 40% greater than those animals on DCA, suggesting that the pituitary response to the experimental conditions described was unique and was not related specifically to increased retention.

There is no evidence in the organic and inorganic distribution of I^{131} in the tissues studied to support the view that organic forms of I^{131} are transferable across cellular membranes or are influenced in passage across membranes by the presence of DCA. Recently Courrier *et al.*, (1951) reported the entry of tagged thyroxine equally into both lobes of the pituitary of the rat two hours following injection. There is a possibility that a more rapid rate of inorganic I^{131} entry into the cells would permit an increased amount of I^{131} to become protein bound directly within the cell.

Franke *et al.*, (1951) reported that adrenalectomized rats fed 2.0% saline as drinking water and given 0.5 mg. DCA daily for 8 weeks exhibited a mean I^{131} concentration ratio almost double that of the latter. When adrenalectomized rats were given 0.5 mg. DCA daily but no saline, the pituitary and thyroid showed I^{131} concentration ratios scarcely different from those rats adrenalectomized but receiving no DCA or saline at all. In this instance it would appear the increased sodium chloride in the diet in the presence of DCA was responsible in adrenalectomized rats for a considerable increase of I^{131} concentration ratios in the pituitary and the thyroid which DCA administration alone could not do. The heightened activity at the pituitary suggests this organ may have been active in mediating this response in the absence of the adrenals.

The increased I^{131} concentration ratios in the pituitary and adrenals here reported after hemithyroidectomy and recently reported (Boatman *et al.*,

1951) in young completely thyroidectomized rats may be due to increased retention of I^{131} resulting from increased antidiuretic activity by the posterior pituitary, increased production of mineral corticoids by the adrenal cortex or disturbances in the interrelationship and antagonistic action of the posterior pituitary antidiuretic hormone and the "salt regulating" hormones of the adrenal cortex.

SUMMARY

Intramuscular DCA or sesame oil was administered to hemithyroidectomized and sham operated rats for thirty days, and the distribution of radioiodine in terms of the I^{131} concentration ratios determined for the thyroid, pituitary, adrenals and blood. The radioiodine trichloroacetic acid soluble and insoluble fractions of these tissues were determined.

While most tissues showed increased I^{131} concentration ratios following DCA injections, the pituitary I^{131} concentration ratios were decreased with DCA, the erythrocytes of the DCA treated animals had an increased per cent of blood radioiodine, and the radioiodine of the pituitary and adrenals of DCA treated hemithyroidectomized animals was largely found in the acid insoluble fraction.

It was concluded the administration of DCA increased retention of I^{131}. The possible mechanism and significance of these findings is discussed.

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NOTES AND COMMENTS

TWO TYPES OF BASOPHILS IN THE RAT PITUITARY: "THYROTROPHS" AND "GONADOTROPHS" VS. BETA AND DELTA CELLS

In a recent paper Purves and Griesbach (1951a), using the periodic acid Schiff (PAS) method for the demonstration of glycoproteins, differentiated two types of pituitary basophils in the rat and called them "thyrotrophs" and "gonadotrophs," respectively. Earlier, we had distinguished between two kinds of basophils on the basis of the affinity of their granules to aldehyde fuchsin (AF) or light green (Halmi, 1950) and, in accordance with the terminology of Romeis (1940), called the AF positive cells beta cells and the ones staining only with light green delta cells. Purves and Griesbach expressed their belief that the beta cells are identical with their "thyrotrophs" and the delta cells with their "gonadotrophs." It is the purpose of this note to clarify further this issue and to point out the excellent agreement between our findings and those of the New Zealand investigators.

There is little reason to doubt that in conditions not involving hypersecretion of thyrotrophin (TSH) delta cell is indeed synonymous with "gonadotroph" and beta cell with "thyrotroph." Both delta cells and "gonadotrophs" were depicted and described as round cells located chiefly in peripheral parts of the adenohypophysis, which are more numerous in the male and, in case of sex hormone deficiency, give rise to the so-called "castration cells." Estrogen induced regression, as seen in the "gonadotrophs," was also reported for the delta cells (Halmi, 1951a; Halmi and Bogdanove, 1951). As to the "thyrotrophs," both they and the beta cells were described as angular cells situated predominantly in central areas of the pars distalis. Purves and Griesbach mention the involution of the "thyrotrophs" under the effect of thyroxin and we made similar observations on the beta cells of rats rendered hyperthyroid with thyroxin (Halmi, 1951a). The New Zealand group reports that low doses of estrogen have no effect on the "thyrotrophs," whereas higher doses lead to their regression. We observed in accordance with this that amounts of estrogen which wiped out the delta cells almost completely in 32–35 days had no influence on the beta cells (Halmi, 1951a; Halmi and Bogdanove, 1951), while higher doses or more prolonged administration led to a decrease in their number and to their eventual extinction (unpublished observations; Clifton, 1951).

Purves and Griesbach regard the hyperplastic and vacuolated basophils (thyroidectomy cells) seen in hypothyroid states as "thyrotrophs" which have lost most of their PAS staining material. In our publications the same cells were classified as delta cells on account of their AF negative granulation and no causal correlation was sought between their appearance and the accompanying drop in the beta cell count. However, recent observations of transitional forms between beta cells and thyroidectomy cells in cases of mild hypothyroidism have convinced us that at least the majority of the hyperplastic "basophils" in states of thyroid hormone deficiency stems from beta cells which have hypertrophied and progressively lost the electiveness of their granules towards AF. Furthermore, we have found that incomplete replacement therapy in thyroidectomized rats partially restores the AF-affinity of the granules in some of the thyroidectomy cells and thus leads to the appearance of large, round and sometimes vacuolated beta cells similar to those described in cold exposed rats (Halmi, 1950). We have also encountered

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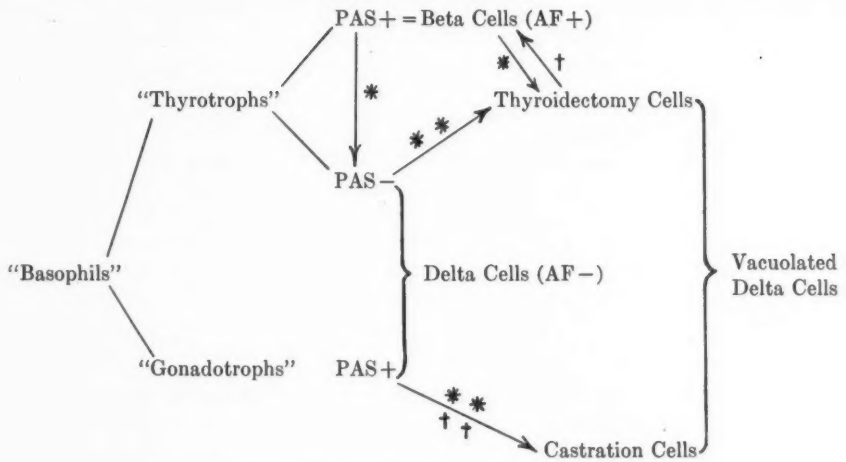

FIG. 1. Relationship between types of pituitary basophils.

✱→ transformation in case of thyroid hormone deficiency.

†→ restitution by thyroid hormone.

✱✱→ transformation through vacuolation.

††→ change in response to sex hormone deficiency.

the large granules described by Purves and Griesbach in the thyroidectomy cells of long term thyroid deficiency. They stain with AF only after prolonged oxidation with hydrogen peroxide and are tinged otherwise in a brilliant shade of green by light green. We believe them to be identical with the fuchsinophilic droplets depicted by Severinghaus (1936) in thyroidectomy cells (Figs. 7 and 9 of his paper).

A diagrammatic presentation of the relationship between "thyrotrophs" and "gonadotrophs" on the one hand and beta and delta cells on the other is given in Fig. 1, which also encompasses their responses to thyroid and sex hormone deficiencies. It illustrates an interpretation of beta and delta cells identical with that presented by Purves and Griesbach (1951b) in a recent note.

The harmonizing experimental results obtained independently by us and the New Zealand group, in conjunction with their histochemical data, suggest the following interpretation of pituitary "basophils" in the rat:

a) The AF positive granular material of the beta cells, which is identical with the PAS staining granules of the "thyrotrophs," is very likely to be TSH. This view was expressed by Purves and Griesbach (1951b) and considered by us in connection with the effects of hypo- and hyperthyroidism on the beta cells (Halmi, 1951a).

b) The enlarged, spherical, poorly granulated and sometimes vacuolated beta cells seen in cases of mild hypothyroidism and exposure to cold are cells which store less TSH owing to an increased rate of discharge.

c) The thyroidectomy cells, which are largely devoid of PAS and AF positive material (not considering the probably non-hormonal coarse droplets), and contain light green staining granules which prompted their classification as delta cells, are "thyro-

trophs" storing little or no TSH but probably secreting it at a considerably augmented rate. The identity of the PAS and aniline blue or light green staining material in the pituitary, which was somewhat overemphasized by Purves and Griesbach, does not hold for these cells.

d) Delta cell granules which give a strong PAS reaction are identical with the "gonadotroph" granules and contain gonadotrophin or at least FSH.

As to the respective merits of the PAS method and the AF-light green technique, a simplified version of the latter having been recently devised and recommended as a routine stain for the pituitary of the rat and the mouse (Halmi 1951b), their appraisal has to take into consideration that in quantitative studies on the anterior hypophysis a differentiation based on tinctorial characteristics of the granules is preferable to one utilizing cytological criteria. AF has the great advantage over PAS of a specific affinity for what appears to be TSH containing granulation. Light green, on the other hand, stains both the granules of the TSH-depleted "thyrotrophs" and the "gonadotrophs." If an AF-light green technique is used the thyroidectomy cells and castration cells have to be distinguished on the basis of the cytological features discussed by Reese, Koneff and Wainman (1943), whereas PAS appears to differentiate clearly between their granules. It can be concluded that the technically somewhat simpler AF-light green method distinguishes adequately between thyrotrophic and gonadotrophic "basophils," except in thyroid hormone deficiency, in which case the supplementary use of the PAS method may be necessary for the proper histological assessment of the functional status of the pituitary.

N. S. HALMI

From the Department of Anatomy
State University of Iowa
Iowa City, Iowa

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